PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Thakker & Ward

Group Art Unit: 1612

Serial No.: 09/974,519

Examiner: Packard, Benjamin J.

Filed: October 10, 2001

Docket No.: 421/32/2

Confirmation No.: 7285

For: COMPOSITIONS AND METHODS FOR ENHANCING PARACELLULAR PERMEABILITY ACROSS EPITHELIAL AND ENDOTHELIAL BARRIERS

* *

DECLARATION OF DHIREN R. THAKKER, PH.D.

PURSUANT TO 37 C.F.R. §1.132

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Sir:

1. My name is Dhiren R. Thakker, Ph.D., and I am the Ferguson Distinguished Professor and Associate Dean, Economic Development and International Partnerships, of the Eshelman School of Pharmacy at the University of North Carolina at Chapel Hill, assignee for the subject U.S. Patent Application Serial No. 09/974,519.

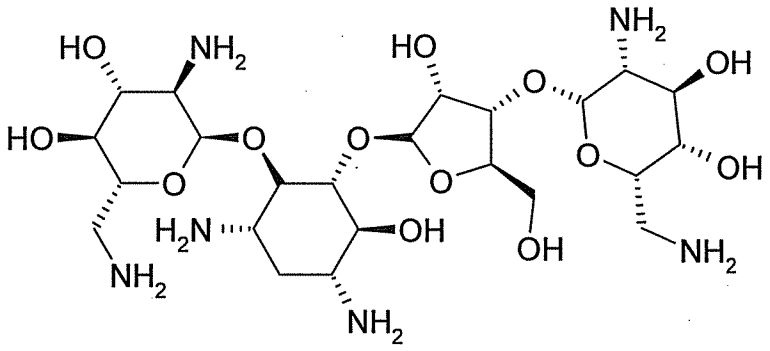
2. A true and accurate copy of my *curriculum vitae*, which evidences my expertise and credentials, is attached herewith and labeled **Exhibit A**.

3. I have had an opportunity to review pending claims 1, 6, and 8 in the subject above captioned U.S. Patent Application Serial No. 09/974,519.

4. I have also reviewed the following documents: the Final Official Action dated April 10, 2008 on the above captioned U.S. Patent Application Serial No. 09/974,519 by the U.S. Patent and Trademark Office (USPTO); the Advisory Action dated December 18, 2008; Cereijido *et al.* (1993) Suppl 17 *J Cell Science* 127-132 ("Cereijido"); and Grunicke *et al.* (1996) 36 *Advan Enzyme Regul* 385-407 ("Grunicke").

5. It is believed that the authors of Cereijido provide no evidence that neomycin treatment of MDCK cells caused inhibition of cellular PLC. Rather, it appears that the authors relied on published work indicating that neomycin is an inhibitor of PLC and used it to provide evidence of PLC's involvement in tight junction formation. However, they fail to recognize that neomycin has multiple cellular/pharmacological actions and that it is not a selective PLC inhibitor. This is demonstrated by Polascik *et al.* (1987) *Biochem J* 243:815-819 and Sipma *et al.* (1996) *Eur J of Pharmacol* 305:207-212 (true and accurate copies of which are provided as **Exhibits B** and **C**, respectively). It is believed that Polascik describes that neomycin can in fact act as a PLC activator (*see* Abstract and page 818, right column, first full paragraph). It is believed that Sipma discloses that neomycin blocked histamine induced Ca^{2+} entry independent of phospholipase C activation, leading the authors to conclude that "neomycin is not a suitable tool to study the effects of messengers generated downstream of phospholipase C activation on receptor mediated and capacitive Ca^{2+} entry in DDT 1 MF-2 cells" (*see* page 211, left column, first full paragraph). These two references are provided just as examples of many reports believed to show that neomycin is not a selective inhibitor of PLC and that its cellular and pharmacological effects are due to multiple mechanisms.

6. Neomycin is a hydrophilic aminoglycoside antibiotic that has the following structure:



7. It is believed that the chemical structure of neomycin, with well over 15 H-bonding sites, several cationic functionalities, and large molecular weight, would prevent it from crossing cell membranes and entering cells. Hence, with my expertise in the area of drug transport mechanisms, it is my opinion that it is highly unlikely that applying neomycin to MDCK cells as described in Cereijido would lead to inhibition of intracellular PLC. In fact, it is believed that Cereijido does not provide any evidence that PLC was inhibited in MDCK cells upon treatment with neomycin.

8. Additionally, it is believed that Cereijido provides no evidence that any effect on TER by thyrotropin-1 releasing hormone (TRH) is mediated by PLC activation. The data in Figure 4 of Cereijido purports to show that TRH treatment of MDCK cells enhances TER by approximately 2-fold over that produced by 1.2 mM Ca^{2+}. It is believed that from this, the authors conclude that TRH activates PLC through TRH receptors, and further conclude that the activated PLC increases TER over that produced by 1.2 mM Ca^{2+} by stimulating the formation of tight junctions beyond those formed by 1.2 mM Ca^{2+} alone.

9. In my opinion, there are several serious flaws in the conclusion of the authors that PLC is involved in the action of TRH. First, upon information and belief, MDCK cells do not express TRH receptors. This is believed to be disclosed in Yeaman *et al.* (1996) *Am J Physiol*, 270:C753-C762, a true and accurate copy of which is provided as **Exhibit D**. On page C755, left column,

last paragraph, this reference states that MDCK cells do not express TRH receptors endogenously (*see also* Figure 2A). Notably, Yeaman was published after Cereijido, and thus it is believed that as of the filing date of the instant U.S. patent application, one of ordinary skill in the art would have understood that TRH could not stimulate PLC in MDCK cells because the cells lack TRH receptors. As such, it is believed that the effect of TRH on increasing TER in MDCK cells reported by Cereijido could not have been due to activation of PLC.

10. Additionally, it is believed that no evidence is presented in Cereijido to show that PLC was in fact activated by TRH treatment of MDCK cells. Upon information and belief, TRH is known to affect multiple cellular mechanisms including, but not limited to modifying HERG channel gating kinetics (*see* Barros *et al.* (1998) *J Physiol* 511:333-346, a true and accurate copy of which is attached as **Exhibit E**).

11. Upon information and belief, there are differences between processes involved in *de novo* formation of tight junctions and processes involved in modifications of formed tight junctions, and the effect of a mediator (such as PLC) on the formation of tight junctions is not the same as its effect on pre-formed tight junctions. As discussed above, it is believed that Cereijido did not provide any evidence that PLC modulation affects the formation of tight junctions in MDCK cells.

12. Further, in my opinion an assumption that if PLC affects the formation of tight junctions, it would also be expected to affect permeability of pre-formed tight junctions is not valid. As shown in Figure 5 of Cereijido, the proposed effect of PLC (and PKC) is to promote fusion of vesicles containing tight junction component proteins with a cell membrane. Cereijido suggests that this occurs by phosphorylation of intracellular vesicles containing tight junction proteins. Upon information and belief, the events leading to the formation of tight junctions is a slow process spanning several hours to days, and involves

trafficking and lodging of various tight junction proteins at appropriate locations in the cell membrane all along the perimeter of the cell.

13. Upon information and belief, the opening and closing of existing tight junctions occurs on a time scale that is several orders of magnitude shorter that the assembly of new tight junctions, and occurs in discrete substructures of the tight junctions (*e.g.*, pores located therein). This is believed to not require complete dismantling and reassembly of the tight junctions.

14. It is further believed that the permeability of pre-formed tight junctions can be modulated by altering the conformation of one or more tight junction proteins. As such, it is believed that the mechanism proposed by Cereijido by which tight junctions are formed is very different from the mechanism by which pre-formed tight junctions transiently open and close to allow passage of small molecules across the spaces enclosed by these junctions. Therefore, it is my opinion that any mechanism involved in the formation of new tight junctions would not teach one of ordinary skill in the art how to modulate the permeability of pre-formed tight junctions.

15. In contrast, the effect of PLC on the permeability of pre-formed tight junctions is believed to occur by de-stabilizing the interactions among the tight junction proteins and/or interactions between the tight junction proteins and the cell membrane. These are believed to be two very different actions. Particularly, the effect of PLC activity on the permeability of pre-formed tight junctions is believed to occur by de-stabilizing the interactions among the tight junction proteins and/or interactions between the tight junction proteins from two adjoining cells. This suggests that modulation of PLC activity would be expected to modify the structure, conformation, and/or organization of tight junction proteins (*e.g.*, actin filament reorganization) to cause the change in the architecture of existing tight junctions, causing them to disrupt or become leaky.

16. Such an example of the effect of PLC inhibition on the actin filament reorganization is provided in Ward *et al.* (2003) *J Pharmaco Exp Ther* 304:689-698, a true and accurate copy of which is attached as **Exhibit F**. Ward shows that inhibition of PLC by hexadecylphosphocholine (HPC) causes disorganization of actin filaments in MDCK cell monolayers, and that this is accompanied by increase in tight junction permeability (*see* Figure 7 of Ward). Ward further shows that when ATP treatment attenuates the inhibition of PLC, caused by another agent (U73122), it also attenuates the decrease in TER and disorganization of actin filaments caused by this agent.

17. It is believed that the comparison of the mechanisms of PLC action proposed by Cereijido and Ward clearly shows that these two are very different targets of possible PLC action and involve mechanisms of action that are believed to be significantly different. The hypothetical model of PLC action on the formation of tight junctions proposed by Cereijido at best suggests that PLC might have an effect on phosphorylation of the membrane of the vesicle carrying tight junction proteins that would be used in the construction of future tight junctions. Even assuming *arguendo* that this is correct with respect to tight junction formation, it is believed that this model would not provide one of ordinary skill in the art with a reasonable expectation that PLC would also act to modify the structure and permeability of pre-formed tight junctions.

18. Upon information and belief, the inhibition of tight junction formation *in vivo* would prevent the epithelial tissue from being formed altogether, which would likely be very toxic to the animal. In contrast, if tight junctions already present in the epithelial tissue are modulated by inhibition of PLC as disclosed in the subject U.S. Patent Application Serial No. 09/974,519, they are not completely destroyed and reformed. Rather, it is believed that the permeability of the tight junctions increases by subtle changes in the structure of tight junction proteins, which changes return to their original state once the PLC inhibition is removed as the PLC inhibitor moves forward in the GI tract due to gastric motility.

19. Thus, the model proposed in Cereijido is believed to have been disproven by the experiments disclosed in the references that were published subsequently to Cereijido, examples of which are attached. As such, it is believed that one of ordinary skill in the art would have understood that the Cereijido model was inapplicable to *in vivo* modulation of tight junction permeability in the intestinal epithelium or other epithelial tissues such as, but not limited to, the blood brain barrier.

20. Additionally, the Patent Office appears to assert that since the intestinal epithelium is continuously creating new tight junctions, an administered PLC inhibitor would be expected to impact formation of at least one tight junction and thus impact paracellular permeability. Upon information and belief, while the intestinal epithelium is constantly turning over with a half life of days, the cells responsible for this turnover are located deep within the intestinal crypts. Epithelial cells are generated in the crypts, which then migrate to the surface of the intestinal epithelium. It is believed that the new tight junctions are formed just as the cells are moving out of the crypts; *i.e.*, in a location that is physically separated from the vilus, the site of drug absorption in the intestinal epithelium. As such, when the cells reach near the tip of the vilus and become available for interactions with drug molecules and their absorption, they have long since formed all of their tight junctions. Therefore, since the formation of new tight junctions occurs in the crypt and not in the vilus where drug absorption occurs, even if PLC inhibitors could inhibit tight junction formation, it is believed that a PLC inhibitor (*e.g.*, a alkylphosphocholine) administered to a subject (*i.e.*, *in vivo*) would not come in contact with cells at a time that they were forming a new tight junction due to the physical separation of the cells in the crypt from the cells on the vilus (*i.e.*, the cells where absorption takes place).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to

be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

Dhiren R. Thakker, Ph.D.

February 4, 2009

Date

Attachments: **Exhibit A-F**

EXHIBIT A

CURRICULUM VITAE
Dhiren R. Thakker

EDUCATION:

Ph.D. (Biochemistry) University of Kansas, Lawrence, KS, 1975
M.S. (Pharmaceutical Chem.) Columbia University, NY, 1972
B.S. (Pharmacy) Bombay University, Bombay, India, 1970

PROFESSIONAL EXPERIENCE

2/96 - current
Ferguson Distinguished Professor of Pharmaceutical Sciences, Division of Molecular Pharmaceutics (formerly Division of Drug Delivery and Disposition), School of Pharmacy (now UNC Eshelman School of Pharmacy)
University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

8//08 – current
Associate Dean, Economic Development and International Partnerships
UNC Eshelman School of Pharmacy

7/98 - 4/08
Associate Dean, Research and Graduate Education
School of Pharmacy (now UNC Eshelman School of Pharmacy)

2/96 -7/05
Joint appointment: Pharmacology
University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

12/95 - 2/96
Visiting Professor
Pharmaceutics Division, School of Pharmacy
University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

3/92 - 11/95
Director, Drug Metabolism Department,
Glaxo Research Institute, Research Triangle Park, NC 27709

7/90 - 2/92
Department Head, Drug Metabolism Department,
Glaxo Research Institute, Research Triangle Park, NC 27709

12/87 - 7/90
Section Head, Bioorganic Mechanisms Section, Drug Metabolism Department,
Glaxo Inc., Research Triangle Park, NC 27709

6/93 - 2/96

EXHIBIT A

Adjunct Professor, Department of Pharmacology, School of Medicine, University of North Carolina, Chapel Hill, NC 27514

11/90 - 2/96
Adjunct Professor, Department of Medicinal Chemistry,
School of Pharmacy, University of North Carolina, Chapel Hill, NC 27514

1/84 - 12/87
Senior Investigator, Laboratory of Molecular Pharmacology, Division of Biochemistry and Biophysics, Center for Drugs and Biologics,
Food and Drug Administration, Bethesda, MD 20892.

9/78 - 1/84
Senior Staff Fellow/Research Chemist, Laboratory of Bioorganic Chemistry,
National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases,
National Institutes of Health, Bethesda, MD 20892

9/76 - 8/78
Staff Fellow, Laboratory of Chemistry, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, MD 20892

9/75 - 8/76
Visiting Fellow, Laboratory of Chemistry, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, MD 20892

MANAGEMENT EXPERIENCE

1. **Led and Managed the Preclinical Drug Metabolism Department at Glaxo Research Institute**
   - The department consisted of a staff of 36 including 19 Senior Scientists with Ph.D. or equivalent experience.
   - The annual operating budget was $6 million, and the capital budget was approximately $1.5 million.
   - The primary responsibility of the department was to conduct non-clinical metabolism, pharmacokinetic and toxicokinetic studies in support of development candidates, and to participate in the discovery and selection of new drug candidates to achieve optimum metabolic and pharmacokinetic profiles.
   - The department also had the responsibility to synthesize radioisotope-labeled compounds for the Research and Development Divisions.

EXHIBIT A

2. **As a member of the Technical Development Team (management team), participated in the coordination of exploratory development programs.**

- The Technical Development Team had representation from Toxicology, Drug Metabolism, Pharmaceutics, Analytical Chemistry, Process Chemistry, Clinical Pharmacology, Regulatory Affairs, and Project Planning.
- The Technical Development Team participated in the formulation of strategies for exploratory development (preclinical through phase I/phase II) of drug candidates and was responsible for the implementation of these strategies.

3. **Interacted with the FDA on the non-clinical ADME issues.**

4. **As a member of the Research Management Committees, participated in the management of Discovery Programs in Cancer, Metabolic Diseases, and Inflammation.**

PROFESSIONAL ACTIVITIES:

Co-Editor, Medicinal Research Reviews, 2001-2006
Editorial Board, J. Pharm. Sci., Since 1997.
Advisory Board, Office of Technology Development, UNC-Chapel Hill
Advisory Board, Office of Business and Economic Development, UNC-Chapel Hill
Advisory Board, Carolina Student Biotechnology Network, UNC, Chapel Hill
Editorial Board, Current Drug Metabolism, 1999-2002.
Editorial Board, Drug Metabolism and Disposition, 1994-1997.
Board of Directors and Scientific Advisory Board, Qualyst Inc. since 2001.
Drug Development Advisory Board, Scios Inc. (subsidiary of J & J), 2001-2005.
Science and Technology Advisory Board, Oread Inc., 1997-2000.
Scientific Advisory Board, Navicyte, 1999-2001.
Awards Committee, Society for Biomolecular Screening (SBS), 2003-2006.
AAPS Taskforce on Drug Discovery Interface, 2005
Founding Member of the Steering Committee, RTP Drug Metabolism Discussion Group, 1999-2002.
Member of the External Review Committee, Graduate Program, School of Pharmacy, University of Washington, Seattle, 2001.
Basic Pharmacology Advisory Committee, Pharmaceutical Manufacturers' Association Foundation, 1994-1996.
Special Emphasis Panel, National Institute of General Medical Sciences, NIH, 1996.

EXHIBIT A

Organizer - Short Course on "Designing Safe Drugs - Integration of Disposition Studies in Drug Discovery and Development", Residential School on Medicinal Chemistry, Drew University

Organizer and Chair– Short Course on "Prodrug Design – Enhanced and Targeted Delivery of Therapeutic Agents" at the 7th North American ISSX Meeting, October 1996, Sand Diego, CA.

Chair - Special Symposium on *"Drug Delivery and Prodrug Technologies"* at the 31st ACS Western Regional Meeting held in October 1995.

Chair - Session on *"Integration of Preclinical ADME Studies in the Preclinical and Clinical Safety Assessment"* at the annual meeting of the Drug Information Association, 1994.

Chair - Session on *"Delivery and Disposition of Peptides and Oligonucleotides-Current Status and Future Challenges"* at the annual meeting of the International Society for the Study of Xenobiotics held in October 1994.

Special reviewer for Geriatric Review Committee, Institute on Aging, 1985.

Reviewer for Investigational New Drug (IND) applications primarily dealing with Interferons and Monoclonal antibodies, 1984-1987.

Reviewer for several leading journals including Nature, Cancer Research, Journal of American Chemical Society, Molecular Pharmacology, Carcinogenesis, Chemico-Biological Interactions, Analytical Biochemistry, Toxicology and Applied Pharmacology, and Chemical Research in Toxicology, Drug Metabolism and Disposition, Pharmaceutical Research, Journal of Pharmaceutical Sciences, Journal of Experimental Therapeutics.

Served as a consultant to Amgen, Amylin Pharmaceuticals, BASF Bioscience, Du Pont Pharmaceuticals, Glaxo Wellcome, ICAgen, Intercardia, Parke Davis, Proctor & Gamble, Synaptics, Triangle Pharmaceuticals, Trimeris, Wyeth, Pozen, Ontogen, Chiron, Scios, *Medivation, Arete, Virobay, Sanofi-Aventis, USVP venture partners (Italicized – consulted during 2007).*

Served as an Expert Witness for Wyeth in Wyeth v. Impax Laboratories, Inc. (deposed – Nov 2007).

HONORS AND AWARDS:

FELLOW, American Association of Pharmaceutical Scientists.

SATO MEMORIAL INTERNATIONAL AWARD presented by the Pharmaceutical Society of Japan in 1987.

PHILLIP NEWMARK AWARD for "Excellence in Biochemical Research" at the University of Kansas in 1974.

DISSERTATION FELLOWSHIP at the University of Kansas in 1975.

GRADUATE HONORS FELLOW at the University of Kansas.

Placed SECOND in Bombay University at the B. Pharm. degree examination.

EXHIBIT A

RESEARCH:

(1) Mechanisms of drug transport across biological barriers
(2) Regulation and modulation of tight junctions in biological barriers
(3) Targeted drug delivery to solid tumors via prodrugs
(4) Gene therapy: in vivo disposition of genes
(5) Stereoselectivity of cytochrome P450 isoenzymes
(6) Metabolic activation of xenobiotics to mutagens and carcinogens

Ph.D.

(1) Development of specific irreversible inhibitors of the enzyme, COMT, which plays an important role in the metabolism of epinephrine, norepinephrine, dopamine, as well as several catecholic endogenous compounds andxenobiotics.
(2) Study of relationship between physico-chemical properties of 8-hydroxyquinolines and their COMT inhibitory activity (Hansch approach).
(3) Affinity chromatography of COMT.
(4) Chemical modification of functional groups on COMT active-site.

M.S. Synthesis of imidazole derivatives as potential chymotryptic agents.

PUBLICATIONS: Co-author of 146 publications which include papers in the peer reviewed journals, review articles, and book chapters (List Attached), co-editors of 2 books, and co-inventor on 6 patents (issued and pending).

ARTICLES

1. Borchardt, R.T. and Thakker, D.: Affinity Labeling of Catechol-O-Methyltransferase with N-Iodoacetyl- 3,5-dimethoxy-4-hydroxyphenylethylamine. Biochem. Biophys. Res. Commun., 54: 1233-1239, 1973.
2. Borchardt, R.T. and Thakker, D.R.: Catechol-O-methyltransferase. 6. Affinity Labeling with N-Iodoacetyl- 3,5-dimethoxy-4-hydroxyphenylalkylamines. J. Med. Chem., 18: 152-158, 1975.
3. Borchardt, R.T. and Thakker, D.R.: Affinity Labeling of Catechol-O-methyltransferase by N-Halonacetyl Derivatives of 3,5-Dimethoxy-4-hydroxyphenylethylamine and 3,4-Dimethoxy-5-hydroxyphylethylamine. Kinetics of Inactivation. Biochemistry, 14: 4543-4551, 1975.
4. Borchardt, R.T., Cheng, C.G. and Thakker, D.R.: Purification of Catechol-O-methyltransferase. 8. Structure-Activity Relationships for Inhibition by 8-Hydroxyquinolines. J. Med. Chem., 63: 69-77, 1975.
5. Bochardt, R.T., Thakker, D.R., Warner, V.D., Mirth, D.B. and Sane, J.M.: Catechol-O-methyltransferase. 8.Structure-Activity Relationships for Inhibition by 8-Hythyltransferase. J. Med. Chem., 19: 558-560, 1976.
6. Brochardt, R.T. Reid, J. R., Thakker, D.R., Liang, Y.O., Wightman, R.W. and Adams, R.N.: Catechol-O-methyltransferase. 9. Mechanisms of Inactivation by 6-Hydroxydopamine. J. Med. Chem., 19: 1201-1209, 1976.
7. Borchardt, R.T. and Thakker, D.R.: Evidence for Sulfhydryl Groups at the Active Site of Catechol-O-methyltransferase. Biochim. Biophys. Acta, 445: 598-609, 1976.

EXHIBIT A

8. Borchardt, R.T. and Thakker, D.R.: Affinity Labeling of Catechol-O-Methyltransferase using N-Haloacetyl Derivatives of 3,5-Dimethoxy-4-hydroxyphenylethylamine and 3,4-Dimethoxy-5-hydroxyphenylethylamine. In Jacoby, W.B. and Wilchek, M. (Ed.): Methods in Enzymology - Affinity Labeling. New York, Academic Press, 1977, Vol. 46, pp. 554-561.
9. Lu, A.Y.H., Levin, W., Vore, M. Conney, A.H., Thakker, D.R., Holder, G. and Jerina, D.M.: Metabolism of Benzo[a]pyrene by Purified Liver Microsomal Cytochrome P-448 and Epoxide Hydrase. In Freudenthal, R.I. and Jones, P.W. (Ed.): Polynuclear Aromatic Hydrocarbons: Chemistry, Metabolism, and Carcinogenesis. New York, Raven Press, 1976, Vol. 1, pp. 115-126.
10. Thakker, D.R., Yagi, H., Lu, A.Y.H., Levin, W., Conney, A.H. and Jerina, D.M.: Metabolism of Benzo[a]pyrene IV. Conversion of (+)-Trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene to the Highly Mutagenic 7,8-Diol-9,10-epoxides, Proc. Natl. Acad. Sci. USA, 73: 3381-3385, 1976.
11. Thakker, D.R., Yagi, H., Akagi, H., Koreeda, M., Lu, A.Y.H., Levin, W., Wood, A. W., Conney, A.M.: Metabolism of Benzo[a]pyrene VI. Stereoselective Metabolism of Benzo[a]pyrene and Benzo[a]pyrene 7,8-Dihydrodiol to Diol Epoxides. Chem.-Biol. Interact., 16: 281-300, 1977.
12. Thakker, D.R., Yagi, H., Levin, W., Lu, A.Y.H., Conney, A.H. and Jerina, D.M.: Stereospecificity of Microsomal and Purified Epoxide Hydrase from Rat Liver: Hydration of Arene Oxides of Polycyclic Hydrocarbons. J. Biol. Chem., 252: 6328-6334, 1977.
13. Yagi, H., Thakker, D.R. Hernandex, O., Koreeda, M. and Jerina, D.M.: Synthesis and Reactions of the Highly Mutagenic 7,8-Diol-9,10-epoxides of the Carcinogen Benzo[a]pyrene. J. Am. Chem. Soc., 99: 1604-1611, 1977.
14. Yagi, H., Thakker, D.R., Mah, H.D., Koreeda, M. and Jerina, D.M.: Absolute Stereochemistry of the Highly Mutagenic 7,8-Diol-9,10-epoxides Derived from the Potent Carcinogen trans-7,8-Dihyroxy-7,8-dihydrobenzo[a]pyrene. J. Am. Chem. Soc., 99: 2358-2359, 1977.
15. Whalen, D.L. Montemarano, J.A., Thakker, D.R., Yagi, H. and Jerina, D.M.: Changes of Mechanism and Product Distributions in the Hydrolysis of benzo[a]pyrene 7,8-Diol-9,10-epoxide metabolites Induced by Changes in pH. J. Am. Chem. Soc., 99: 5522-5524, 1977.
16. Wood, A.W., Chang, R.L., Levin, W., Yagi, H. Thakker, D.R., Jerina, D.M., and Conney, A.H.: Differences in Mutagenicity of the Optical Enantiomers of the Diastereomeric Benzo[a]pyrene 7,8-Diol-9,10-epoxides. Biochem. Biophys. Res. Commun., 77: 1389-1396, 1977.
17. Wood, A.W., Levin, W., Ryan, D., Thomas, P.E., Yagi, H., Mah, H.D., Thakker, D.R., Jerina, D.M., and Conney, A.H.: High Mutagenicity of Metabolically Activated Chrysene 1,2-Dihydrodiol: Evidence for Bay Region Activation of chrysene. Biochem. Biophys. Res. Commun., 78: 847-854. 1977.
18. Levin, W., Wood, A.W., Lu, A.Y.H., Ryan, D., West, S., Thakker, D.R., Yagi, H., Jerina, D.M., and Conney, A.H.: Role of Purified Cytochrome P-448 and Epoxide Hydrase in the Activation and Detoxification of Benzo[a]pyrene. In Jerina, D.M. (Ed.): Drug Metabolism Concepts. Washington, D.C., American Chemical Society, 1977, ACS Symposium Series 44, pp. 99-126.

EXHIBIT A

19. Jerina, D.M. Lehr, R., Shaefer-Ridder, M., Yagi, H., Karle, J.M., Thakker, D.R., Wood, A.W., Lu, A.Y.H., Ryan, D., West, S., Levin, W., and Conney, A.H.: Bay Region Epoxides of Dihydrodiols: A Concept which Explains the Mutagenic and Carcinogenic Activity of Benzo[a]pyrene and Benzo[a]amthraceme. In Hiatt, H., Watson, J.D. and Winsten, I. (Ed.): Origins of Human Cancer. New York, Cold Spring Harbor Laboratories, 1977, PP. 639-658
20. Levin, W., Lu, A.Y.H., Ryan, D., Wood, A.W., Kapitulnik, J., West, S.,Huang, M.T., Thakker, D.R., Holder, G., Yagi, H., Jerina, D.M. and Conney, A.H.: Properties of the Liver Microsomal Monooxygenase System and Epoxide Hydrase: Factors Influencing the Metabolism and Mutagenicity of Benzo[a]pyrene. In Hiatt, H., Watson, J.D., and Winsten, I. (Ed.): Origins of Human Cancer. New York, Cold Spring Harbor, Cold Spring Harbor Laboratories, 1977, pp.659-682.
21. Bresnick, E., Stoming, T.A., Vaught, J.B., Thakker, D.R., and Jerina, D.M.: Nuclear Metabolism of Benzo[a]pyrene and of (+)-trans-7,8-Dihydroxy-7,8-dihydrobenzo[a]pyrene. Comparative Chromatographic Analysis of Alkylated DNA. Arch. Biochem. Biophys., 183: 31-37, 1978.
22. Lehr, R.E., Yagi, H., Thakker, D.R., Levin, W., Wood, A.W., Conney, A.H. and Jerina, D.M.: The Bay Region Theory of Polycyclic Aromatic Hydrocarbon Induced Carcinogenicity. In Freudenthal, R.I. and Jones, P.W. (Ed.): Polynuclear Aromatic Hydrocarbons: Chemistry, Metabolism and Carcinogenesis. New York, Raven Press, Vol. 2., pp.231-241.
23. Thakker, D.R., Levin, W., Stoming, T.A., Conney, A.H., and Jerina, D.M.: Metabolism of 3-Methylcholanthrene by Rat Liver Microsomes and a Highly Purified Monooxygenase System With and Without Epoxide Hydrase. In Freudenthal, R.I., and Jones, P.W.(Ed.): Polynuclear Aromatic Hydrocarbons: Chemistry, Metabolism and Carcinogenesis. New York, Raven Press, 1978, Vol. 2., pp.253-264.
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PATENTS AND PATENT APPLICATIONS

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1. Method of Screening Candidate Compounds for Susceptibility to Oxidative Metabolism, US 6,312,917.
2. PLC-based Absorption Enhancers, patent pending
3. Amide Phospholipid-like Compounds as Drug Absorption Enhancers, patent pending.
4. Pharmacokinetic Tool and Method for Predicting Metabolism of a compound in a Mammal, WO 02/10746
5. High Throughput Reactive Oxygen Species-Based Cytochrome P450 Inhibition Assay. US Patent Application filed.
6. Methods and Kits for Determining Metabolic Stability of Compounds. US Patent Application filed.
7. Direct Activation of Human Phospholipase Cß3 (HPLCß3) by U73122 in Dodecylmaltocide (DDM) Mixed Micelles via Alkylation at Cysteine Residues, US Patent Application filed.

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42. Sinhababu, A. K., Boehlert, C., Levesque, D., Gan, L.-S., and Thakker, D. R.: "Mechanism of Inhibition of Ribonucleoside Diphosphate Reductase from Rabbit Bone Marrow by 2'azido-2'-deoxycytidine-5'diphosphate." Proceedings of the American Association for Cancer Research, 32, 12, 1991.
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44. Thakker, D. R., Patrick, M. A., Prasad, K. B., Boehlert, C., and Mirsadeghi, S.: "Effect of the Fluoro Substituent on the Regio- and Stereoselectivity of Cytochromes P-450-catalyzed Oxidation of Polycyclic Aromatic Hydrocarbons." Proceedings of the Third International Meeting of the International Society for the Study of Xenobiotics (ISSX),302, 1991.
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46. Sinhababu, A.K., Levesque, D.L., Ghate, J., and Thakker, D.R.: "Inactivation of Ribonucleoside Diphosphate Reductase from Rabbit Bone Marrow by Nucleotide 2',3'-Dialdehydes." Proceedings of the American Association for Cancer Research, 33, 1992.

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99. Bourdet, D.L., Ming, X. and Thakker, D.R.: Do Organic Cation Transporters (Slc22a1-5) Have A Role In The Intestinal Absorption Of Hydrophilic Cationic Drugs?, AAPS Workshop On Drug Transporters – From Bench To Bedside, 2005.

100. Boudet, D. L. and Thakker, D. R., Intestinal Transport of Hydrophilic Cations: A Kinetic Modeling Approach to Elucidate the Role of Uptake and Efflux Transporters and Paracellular vs. Transcellular Transport in the Absorptive Transport of Ranitidine, AAPS, 2005.

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102. Klein R, Boudon, D., and Thakker, D. R. Paracellular Permeability Enhancers U73122 and Alkylphosphocholines Inhibit Phospholipase C β Isozymes in A Cell-Free System, AAPS, 2005.

103. Tippin T. K., Cummings, C. A., and Thakker, D. R. A Cell Culture Model to Evaluate Local and Systemic Toxicity of Intestinal Paracellular Permeability, AAPS, 2005.

104. Ming, X., Hong, S., and Thakker, D. R. Evidence for Cell Membrane Damage by EGTA During Ca^{+2}-switch Experiment in Caco-2 Cells to assess Paracellular Transport of Compounds, AAPS, 2005.

105. Ming, X., Bourdet, D. L., and Thakker, D. R. Gene Expression Profile of Human Organic Cation Transporters along the Gastrointestinal Tract and in Caco-2 Cells, AAPS, 2005.

106. Ming, X., and Thakker, D. R. Expression and Functional Activity of the Heteromeric Organic Solute Transporter alpha-beta in Caco-2 Cells, AAPS, 2006

107. Proctor, W., Bourdet, D. L., and Thakker, D. R. A Kinetic Modeling Approach to Determine the Role of Uptake Transporters and Relative Contribution of Paracellular vs. Transcellular Transport in the Absorptive Transport of Metformin in Caco-2 Cells, AAPS, 2006.

108. Mowrey, B. and Thakker, D.R. CYP3A-mediated Metabolism of Terfenadine During Absorptive Transport across Intestinal Tissue from P-gp-deficient and P-gp-competent Mice., AAPS , 2006

109. Ming, X., Knight, B., and Thakker, D. R. Involvement of Multidrug Resistance-associated Proteins (MRPs) in Intestinal Transport of the Anti-allergic Drug Fexofenadine, AAPS Transporter Meeting, 2007.

110. Proctor, W., and Thakker, D. R. Saturable Absorptive Transport of Metformin across Caco-2 Cell Monolayers Occurs Predominantly via a Novel Paracellular Transport Mechanism, AAPS Transporter Meeting, 2007

111. Knight, B., and Thakker, D. R. Transport/Metabolism Interplay for Loperamide and Terfenadine in the CYP3A-Expressing Caco-2 Cell Model, AAPS Transporter Meeting, 2007.

112. Klein, R. and Thakker, D. R. A role for phospholipase Cβ in the regulation of paracellular permeability in a human intestinal epithelial cell line. *FASEB J.* 21:729.8 2007.

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113. Klein, R.R., Bourdon, D.M., Wagner, C.D., White, W.L., Williams, J.D., and Thakker, D.R. Direct activation of human phospholipase Cβ3 (hPLCβ3) by U73122 in dodecylmaltoside (DDM) mixed micelles via alkylation at cysteine residues. *FASEB J.* 21:729.9 2007.

INVITED LECTURES

1. Clinical Society of Washington, Rockville, MD., 1976.
2. Department of Pharmacology, George Washington University, Washington, D.C., 1977.
3. Department of Chemistry, George Washington University, Washington, D.C., 1977.
4. Syntex Pharmaceutical Co., Palo Alto, CA., 1978.
5. Departments of Medicinal Chemistry and Biochemistry, University of Kansas, Lawrence, KS, 1978.
6. Environmental Health Chemistry Symposium, American Chemical Society, Washington, DC., 1979.
7. 39th International Congress of Pharmaceutical Sciences, Brighton, U.K., 1979.
8. Department of Chemical Technology, Bombay University, Bombay, India, 1980.
9. Cancer Research Institute, Bombay, India, 1980.
10. Bristol Myers Laboratories, Syracuse, NY., 1980.
11. Laboratory of Developmental Pharmacology, National Institute of Child Health and Human Development, NIH, Bethesda, MD., 1981.
12. Laboratory of Biochemistry and Metabolism, NIADDK, NIH, Bethesda, MD., 1981.
13. International Symposium on Biological Reactive Intermediates, Gilford, Surrey, U.K., 1981.
14. Interx-Merck Pharmaceutical Co., Lawrence, KS., 1982.
15. Smith Kline Beckman, Philadelphia, PA., 1982.
16. Indian Pharmaceutical Association, Bombay, India, 1983.
17. Cancer Research Institute, Bombay, India, 1983.
18. National Center for Drugs and Biologics, FDA, Bethesda, MD., 1983.
19. Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, KS., 1984.
20. Smith Kline Beckman, Philadelphia, PA., 1985.
21. Cancer Research Institute, Bombay, India, 1986.
22. Central Drug Research Institute, Lucknow, India, 1986.
23. Bhabha Atomic Research Center, Bombay, India, 1986.
24. Hochest Pharmaceuticals, Bombay, India, 1986.
25. 6th International Catecholamine Symposium, Jerusalem, Israel, 1987.
26. SATO Memorial International Award Lecture at the 107th Annual Meeting of the Pharmaceutical Society of Japan, Kyoto, Japan, 1987.
27. Nagoya City University, Facility of Pharmaceutical Sciences, Nagoya, Japan, 1987.
28. Tohuku University, Research Institute for Tuberculosis and Cancer Sendai, Japan, 1987.
29. Keio University School of Medicine Tokyo, Japan, 1987.
30. Tokyo College of Pharmacy, Tokyo, Japan, 1987.
31. National Institute of Hygienic Sciences, Tokyo, Japan, 1987.

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32. Department of Chemical Technology, Bombay University, Bombay, India, 1987.
33. Laboratory of Bioorganic Chemistry, NIDDk, Bethesda, MD., 1987.
34. Carcinogen Risk Assessment, Banbury Center, Cold Spring Harbar, 1987.
35. Pharmaceutical Institute, School of Medicine, Keio University, Tokyo, Japan, 1988.
36. Tokyo College of Pharmacy, Tokyo, Japan, 1988.
37. Dept. of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan, 1988
38. Dept. of Medicinal Chemistry, University of North Carolina, 1989.
39. Dept. of Medicinal Chemistry, University of North Carolina, 1990 (presented 3 lectures in a Course on Drug Metabolism).
40. Dept. of Medicinal Chemistry, University of North Carolina, 1991 (presented 3 lectures in a Course on Drug Metabolism).
41. Dept. of Pharmacology, University of North Carolina, 1991.
42. Annual Meeting of the PMA Drug Metabolism Section, New Orleans, 1991.
43. 25th Annual Higuchi Symposium, Lake of the Ozarks, 1992.
44. Enz Lecture, Dept. of Pharmaceutical Chem., University of Kansas, 1992
45. Dept. of Medicinal Chemistry, University of North Carolina, 1992 (presented 3 lectures in a course on Drug Metabolism).
46. Dept. of Medicinal Chemistry, University of North Carolina, 1993 (presented 3 lectures in a course on Drug Metabolism).
47. Dept. of Chemistry, Catholic University, Washington, D.C., 1993.
48. Lab of Bioorganic Chemistry, NIDDK, NIH, 1993.
49. Chaired a session on the "Integration of Preclinical ADME studies in the Preclinical and Clinical Safety Assessment" at the National Meeting of Drug Information Associatoin, at Wahington, D.C., 1994.
50. Chaired a session on "Delivery and Disposition of Peptides and Oligonucleotides-Current Status and Future Challenges" at the annual meeting of the International Society for the Study of Xenobiotics (ISSX), Raleigh, NC, October 1994.
51. Chaired a Special Symposium on "Drug Delivery and Prodrug Technologies" at the 31st ACS Western Regional Meeting, San Diego, CA, October 1995.
52. Conference on "Lead Generation and Optimization", Princeton, NJ, September, 1996.
53. Organized a short course presented a talk on Prodrugs at the 7th North American ISSX Meeting, San Diego, October 1996.
54. Winter Conference on "Medicinal and Bioorganic Chemistry", Steamboat Springs, Colorado, January, 1997.
55. Third International Symposium on Innovations in Pharmaceutical Sciences and Technology", Ahmadabad, India, February 1997.
56. S.N.D.T. College of Pharmacy, Bombay University, Bombay, India, February, 1997.
57. Tata Institute for Cancer Research, Bombay, India, February, 1997.
58. Amylin Pharmaceuticals, San Diego, CA, April, 1997
59. Merck Research, West Point, PA, April, 1997.
60. Proctor & Gamble, Cincinnatti, OH, June, 1997.
61. AAPS Southeast Regional Meeting, Research Triangle Park, NC, June, 1997.
62. Drew University, (faculty), Princeton, NJ, July, 1997.
63. DuPont Merck, Newark, DE, July 1997.
64. Formulations and Drug Delivery, ACS Conference, San Diego, CA, October 1997.

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65. Glaxo wellcome, Inc. Pharmaceutics Division, RTP, NC, January, 1998.
66. Swiss Chemical Society – Minisymposium on Oral Drug Delivery, Basel Switzerland, May, 1998.
67. University of Leuven, Workshop on Industry – Academic Collaborations, Leuven, Belgium, May 1998.
68. Glaxo Wellcome, Inc., Biomet Division, Ware, U.K., May, 1998.
69. AAPS Western Regional Meeting, San Francisco, CA, May, 1998.
70. AAPS Southeastern Regional Meeting, RTP, NC, June, 1998.
71. 28th Annual Gordon Research Conference on Drug Metabolism, Plymouth, NH, July, 1998.
72. Drew University, (faculty), Princeton, NJ, July, 1998.
73. Pfizer, Inc., Groton, CT, July, 1998.
74. 74, Bristol-Meyer Squibb, Inc., Wallingford, CT, July, 1998.
75. School of Pharmacy, University of Michigan, Ann Arbor, MI, September, 1998.
76. Higuchi Research Seminar, Lake of Ozarks, MO, March, 1999.
77. Glaxo Wellcome, Pharmaceutics Dept., RTP, NC, March, 1999.
78. BASF, Boston, MA, June, 1999.
79. Parke Davis, Inc., Ann Arbor, MI, July, 1999.
80. Novartis, East Hanover, NJ, September, 1999.
81. Institute for Innovative Research, (faculty), San Diego, CA, December, 1999.
82. Agouron Pharmaceuticals, San Diego, CA, December 1999.
83. Glaxo Wellcome (Pharmaceutics), RTP, NC, March 2000.
84. Laboratory of Biorganic Chemistry, NIDDK, NIH, Bethesda, MD, May 2000
85. Pratt Fellowship Program, NIH, Bethesda, MD, May 2000
86. Wyeth-Ayerst, Pearl River, N.Y., June 2000
87. Drew University (faculty), Princeton, NJ, June 2000
88. Chiron, CA, 2001.
89. Roche, Palo Alto, CA, 2001
90. Guilford Pharmaceuticals, Baltimore, MD, 2001
91. Drew University (faculty), Princeton, NJ, 2001
92. Drew University (faculty), Course Organizer, priceton, NJ, 2001
93. Transform Pharmaceuticals, Boston, MA, 2001
94. Bristol-Myers Squibb, Princeton, NJ, 2001
95. Albany Biolomolecules, Albany, NY, 2001
96. Lilly Pharmaceuticals, Indianapolis, IN, 2001
97. 3-D Pharmaceuticals, Philadelphia, PA, 2001
98. Pfizer, Ann Arbor, MI, 2002
99. Gilford Pharmaceuticals, Baltimore, MD, 2002
100. Pfizer (Agouron), San Diego, CA, 2002
101. Serono Pharmaceuticals, Soreno, Switzerland, 2002
102. Avantis, Frankfurt, Germany, 2002
103. Bristol Myers Sqibb, Lawrenceville, NJ, 2002
104. Avantis, Pearl River, NJ, 2002
105. Roche, Nutley, NJ, 2002
106. Pfizer, Groton, CT, 2002

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107. GPEN, Detriot, MI, 2002
108. GlaxoSmithKline, Philadelphia, PA, 2002
109. Arena Pharmaceuticals, San Diego, CA, 2002
110. Drew University, Newark, NJ, 2003
111. Novartis, Jolla CA, 2003
112. AstraZeneca, Boston, MA, 2003
113. Fibrogen, San Francisco, CA 2003
114. ADMET-1, San Diego, CA 2004
115. AstraZeneca, San Diego, CA 2004
116. LabFusion, Boston, Ma 2004
117. Millennium, Cambridge, MA 2004
118. Schering-Plough, Kenilworth, NJ 2004
119. Neurocrine, San Diego, CA 2004
120. Abbott, Abbott Park, IL 2004
121. Pfizer, Groton, CT, 2005
122. ICOS, Bothell, WA, 2005
123. SCIOS, Inc. San Francisco, CA, 2005
124. Biogen, Cambridge, MA 2005
125. Genzyme, Framingham, MA 2005
126. Sunesis, San Francisco, CA 2005
127. Drew University, Newark, NJ 2005
128. Amgen, Boston, MA, 2005 "Probing the Intestinal Epithelial Barrier: A Tortuous Journey"
129. Pediatric Pharmacology Research Unit Network Meeting, Washington, D. C., 2005 "Implementing In Vitro Drug Disposition Studies to Improve Pediatric Therapy – Challenges and Opportunities"
130. Cambridge Healthcare Institute – Mastering Medicinal Chemistry/ADME In-depth, San Francisco, CA, 2006 "ADME In-depth: A Medicinal Chemistry Perspective" (Lead Lecture)
131. Scitech Center, Mumbai, India, 2006 "Integration of ADME in Drug Discovery: Past, Present, and Future"
132. Indian Drug Manufacturers' Association Meeting – Bioavailability and Bioequivalence, Mumbai, India, 2006. "Integration of ADME in Drug Discovery: Past, Present, and Future"
133. PERD Center, Ahmedabad, India, 2006 "Integration of ADME in Drug Discovery: Past, Present, and Future" "Probing the Intestinal Epithelial Barrier: A Tortuous Journey"
134. Drew University, Madison, NJ, 2006. "Use of Preclinical Pharmacokinetics and In Vitro Models to Assess Problems of Poor Oral Bioavailability" (with Kim Brouwer); "Role of Intestinal and Hepatic Metabolism in Drug Clearance – Chemical Strategies to Optimize Metabolic Clearnce"
135. Genentech, San Francisco, CA, 2006. "Use of Preclinical Pharmacokinetics and In Vitro Models to Assess Problems of Poor Oral Bioavailability" (with Kim Brouwer); "Role of Intestinal and Hepatic Metabolism in Drug Clearance – Chemical Strategies to Optimize Metabolic Clearnce"

EXHIBIT A

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137. Bristol Myers Squibb, NJ, 2006. Interplay of Transport and Metabolism in the Intestinal Epithelium

138. Nektar Pharmaceuticals, Mobile, Alabama/San Diego, CA, 2007; "Use of Preclinical Pharmacokinetics and In Vitro Models to Assess Problems of Poor Oral Bioavailability" (with Kim Brouwer); "Role of Intestinal and Hepatic Metabolism in Drug Clearance – Chemical Strategies to Optimize Metabolic Clearnce"

139. Molecular Medicine Tri-Conference – ADMET short course, San Francisco, CA, February, 2007. Lead Speaker - *In Vitro* ADME Assays: Strategic Application to Drug Discovery

140. New England Drug Metabolism Discussion Group Symposium, Boston, MA, April 2007. "Drug Metabolism-Pharmacokinetics-guided (DM/PK-guided) Lead Optimization: A Historical Perspective of a Paradigm Shift"

141. Bayer Pharmaceuticals, Dusseldorf, Germany, January 2008 "Use of Preclinical Pharmacokinetics and In Vitro Models to Assess Problems of Poor Oral Bioavailability" (with Kim Brouwer); "Role of Intestinal and Hepatic Metabolism in Drug Clearance – Chemical Strategies to Optimize Metabolic Clearnce"

142. Molecular Medicine Tri-Conference – ADMET short course, San Francisco, CA, March, 2008.

143. Lead Speaker – "*In Vitro* ADME Assays: Strategic Application to Drug Discovery"

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Neomycin cannot be used as a selective inhibitor of inositol phospholipid hydrolysis in intact or semi-permeabilized human platelets

Aminoglycosides activate semi-permeabilized platelets

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High concentrations of neomycin (2–10 mM) inhibited aggregation, but not shape change, of intact platelets by collagen, ADP and the Ca^{2+} ionophore, A23187, the last two studies being carried out in the presence of the cyclo-oxygenase inhibitor indomethacin. In contrast, over the same range of concentrations neomycin inhibited both aggregation and shape change induced by thrombin. Under these conditions activation of platelets by collagen and by thrombin, but not by A23187 or by ADP, is believed to be dependent on the hydrolysis of membrane inositol phospholipids. These data therefore suggest that the inhibitory action of neomycin on intact platelets is not related to its previously reported inhibitory effect on phosphoinositide metabolism. The selective inhibition of thrombin-induced shape change indicates a second site of action of neomycin on intact platelets. On platelets rendered semi-permeable with saponin, neomycin and a second aminoglycoside antibiotic, streptomycin (each 0.06–2 mM), stimulated secretion and aggregation responses. These effects were inhibited by indomethacin and by EGTA. Activation of semi-permeabilized platelets by neomycin is associated with the formation of inositol phosphates and phosphatidic acid, indicating activation by phospholipase C. This effect is also inhibited by indomethacin, implying that it is secondary to the formation of prostaglandins and endoperoxides. These results are discussed in the context of the use of neomycin as a selective inhibitor of polyphosphoinositide metabolism.

INTRODUCTION

Receptor-stimulated hydrolysis of membrane inositol phospholipids has now become recognized as a major transmembrane signalling pathway within the body (for review see [1] and [2]). The hydrolysis of phosphatidylinositol 4,5-bisphosphate generates inositol trisphosphate and 1,2-diacylglycerol, both of which appear to function as second messengers. Inositol trisphosphate stimulates the release of Ca^{2+} from non-mitochondrial stores within the cell, and 1,2-diacylglycerol activates a Ca^{2+} phosphatidylserine-dependent protein kinase, named protein kinase C [1,2]. In the human platelet a variety of agonists stimulate the hydrolysis of phosphatidylinositol bisphosphate, including thrombin [3], collagen [4] and thromboxanes [4], and thus induce the formation of inositol trisphosphate and 1,2-diacylglycerol. These two second messengers are thought to act in synergy to produce secretion and aggregation responses in the platelet [5]. In contrast, other stimuli are believed to activate platelets independently of inositol phospholipid metabolism. ADP at concentrations which produce a similar degree of platelet activation to thrombin does not stimulate inositol phospholipid hydrolysis [6], whereas the Ca^{2+} ionophore A23187, in the presence of cyclo-oxygenase inhibitors, activates platelets by the mobilization of Ca^{2+} [7].

The aminoglycoside antibiotic neomycin binds strongly and selectively to polyphosphoinositides relative to other phospholipids [8–10], and has therefore been used as a potential and relatively specific inhibitor of inositol phospholipid metabolism. For example, the introduction of neomycin (0.01–0.1 mM) into semi-permeabilized mast cells inhibits secretion induced by guanosine 5′-[α-thio]triphosphate, a process thought to be brought about by the hydrolysis of inositol phospholipids [11]; similarly, the addition of neomycin (0.4–10 mM) to the cytoplasmic side of the plasmic membrane in sea-urchin eggs inhibits Ca^{2+}-mediated hydrolysis of inositol phospholipids and subsequent exocytosis [12]. It was necessary in those studies to permeabilize the surface membrane in order to allow the polar neomycin access to the inner leaflet of the plasma membrane, where the polyphosphoinositides are located. There is also a single report, however, showing that at higher concentrations neomycin (2 mM) is inhibitory on intact cells, i.e. neomycin inhibits thrombin-stimulated inositol phospholipid metabolism and cellular proliferation in hamster fibroblasts [13].

Neomycin binds to polyphosphoinositides at concentrations of 0.01–0.1 mM upwards [9,10], and has been shown to inhibit phospholipase C, phospholipases A and phosphatidylinositol 4-monophosphate kinase at these concentrations, using subcellular fragments of brain [14,15], kidney [16,17], ear [18] and erythrocyte [19] membranes. At higher concentrations, neomycin also binds to other phospholipids, and has been shown to inhibit phosphatidylinositol kinase [19], the binding of Ca^{2+} to surface membranes, and Ca^{2+} entry [14]. Other aminoglycosides, including gentamycin and streptomycin, also bind to polyphosphoinositides, but they are less potent than neomycin in this action [17,18]. The

present study investigates whether neomycin selectively inhibits inositol phospholipid hydrolysis in human platelets, and can therefore be used to define further the role of this pathway in platelet activation.

METHODS

Blood (50 ml), anti-coagulated with citrate (15%, except for experiments carried out with platelet-rich plasma, when 3% was used), was taken on the day of the experiment from volunteers who had denied taking aspirin in the previous 2 weeks [3]. Platelet-rich plasma was prepared by centrifuging at 200 *g* for 20 min, and was labelled with 10 μCi of [^{3}H]5-hydroxytryptamine for 1 h at room temperature. Prostacyclin (100 ng) was added, and the platelet-rich plasma was centrifuged at 1000 *g* for 10 min; prostacyclin increases cyclic AMP, and thus prevents aggregation during centrifugation [20]. The platelet-poor plasma was discarded, and 5 ml of 'intracellular' or 'extracellular' (see below) buffer was added to the platelet pellet and also discarded; this ensures the near-complete removal of residual amounts of plasma, which are known to interfere with the action of saponin [21]. The platelets were then resuspended at a concentration of 2×10^8–3×10^8/ml in either 'intracellular' buffer [composition (mM): KCl 140, NaH_2PO_4 0.42, $MgCl_2$ 1, glucose 1, $NaHCO_3$ 11.9, Hepes 5; adjusted to pH 7.35] [20] or 'extracellular' buffer [composition (mM): NaCl 134, $NaHCO_3$ 12, KCl 2.9, Na_2HPO_4 0.34, $MgCl_2$ 1, glucose 5, Hepes 5, adjusted to pH 7.4]. These buffers were designed to mimic the intracellular [22] or extracellular ionic environments found *in vivo*. The platelets were then left at room temperature for 30 min to allow the action of prostacyclin to wear off. In some experiments platelets were prelabelled with [^{32}P]P_i (1 mCi/3 ml) or [^{3}H]inositol (66 μCi/ml) for 1 h and 3 h respectively in extracellular buffer, and washed in the presence of prostacyclin (1 μg) before experimentation.

Experiments were carried out in a Born lumiaggregometer at 37 °C with stirring at 600 rev./min; the platelets (0.5 ml final volume) were prewarmed to 37 °C for 2 min before experimentation. The following time cycles were used: neomycin, streptomycin, indomethacin and EGTA were given at the start of the experiment, and saponin, thrombin, collagen, ADP or A23187 was added 60 s later, and the mixture was left for a further 60 s. The reaction was then stopped by transferring to an equal volume of 6% (v/v) glutaraldehyde for measurements of [^{3}H]5-hydroxytryptamine release [23], by transferring to 1.88 ml of chloroform/methanol (1:2, v/v) for analyses of phosphatidic acid [24], or by transfer to chloroform/methanol/HCl (50:100:1, by vol.) for analyses of inositol phosphates. Phosphatidic acid was separated from other phospholipids by t.l.c. and measured by scintillation counting [24]. Total inositol phosphates were separated on Dowex anion-exchange columns [3,4]. Inositol and glycerophosphoinositol were eluted together with 16 ml of 60 mM-ammonium formate/5 mM-sodium tetraborate, and inositol mono-, bis- and tris-phosphates were also eluted together with 16 ml of 800 mM-ammonium formate/0.1 M-formic acid. Light-transmission was recorded throughout the experiment and was displayed on a chart recorder in order to monitor aggregation. The concentration of saponin used in these studies was estimated as described previously by measuring the leakage of ATP by using luciferin–luciferase [21]. Results are shown as means ± S.E.M. from at least three separate experiments. Statistical comparisons were made by using a Wilcoxon Rank Test and by Student's *t* test.

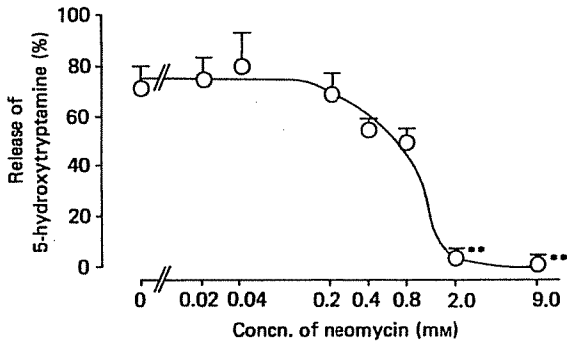

Fig. 1. Concentration–response curve for neomycin inhibition of 5-hydroxytryptamine secretion by thrombin from washed platelets

Washed platelets, prelabelled with [^{3}H]5-hydroxytryptamine, were incubated with various concentrations of neomycin for 1 min before challenge with a just submaximal concentration of thrombin (0.06 unit/ml) for a further 1 min. [^{3}H]5-Hydroxytryptamine secretion was measured as described in the Methods section. Results are shown as means ± S.E.M. for four separate experiments; $^{**}P < 0.01$ relative to thrombin alone.

Neomycin sulphate, streptomycin sulphate, indomethacin, thrombin, ADP and A23187 were purchased from Sigma, Poole, Dorset, U.K. Collagen was from Horm-Chemie, Munich, Germany. [^{3}H]5-Hydroxytryptamine, [^{32}P]P_i and [^{3}H]inositol were from Amersham. All other reagents were of analytical grade.

RESULTS

Intact platelets

On its own, neomycin has no observable action on washed platelets. In contrast, relatively high concentrations of neomycin (2–10 mM) inhibited secretion (Fig. 1), shape change and aggregation (Fig. 2*a*) induced by a concentration of thrombin which was just sub-maximal for aggregation. On the other hand, neomycin inhibited aggregation, but not shape change, induced by the Ca^{2+} ionophore A23187, in the presence of indomethacin. Similarly, on platelets suspended in platelet-rich plasma, neomycin (2–10 mM) inhibited aggregation, but not shape change, induced by collagen or by ADP, the latter experiments also being carried out in the presence of indomethacin (10 μM) (Fig. 2*b*).

Semi-permeabilized platelets

Experiments on platelets rendered semi-permeable with saponin were carried out in a buffer designed to mimic more closely the intracellular ionic environment of the platelet [22]. The concentration–response curves for secretion and aggregation responses to thrombin obtained by using this 'intracellular' buffer (for composition see the Methods section) were similar to those obtained with the 'extracellular' buffer (results not shown).

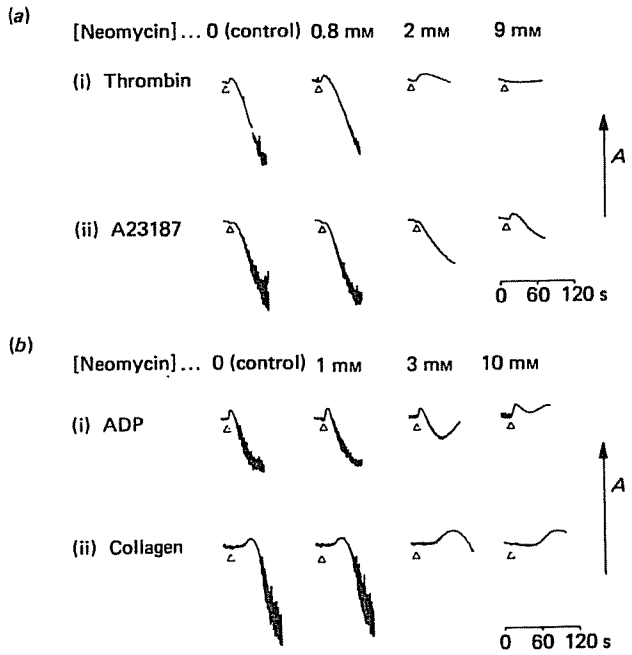

Fig. 2. Increasing concentrations of neomycin inhibit platelet aggregation by thrombin, collagen, A23187 and ADP

Platelets were incubated with various concentrations of neomycin for 1 min before challenge with a just submaximal concentration of agonist for a further 1 min. Indomethacin (10 μM) was present in the experiments involving A23187 and ADP. An increase in light transmission represents the change in shape of the platelets from a disc to a sphere with pseudopodia; a decrease reflects platelet aggregation. △ shows when the agonist was administered, and the decrease in *A* at this time point is a dilution artifact. The results are shown as typical traces from one experiment, which is representative of three others. (*a*) Washed platelets were challenged with either (i) thrombin (0.06 unit/ml) or (ii) A23187 (300 nM). (*b*) Platelets suspended in plasma were challenged with either (i) collagen (2 μg/ml) or (ii) ADP (2 μM).

With platelets rendered permeable with saponin, relatively low concentrations of neomycin (0.06–2 mM) stimulated secretion and aggregation (Figs. 3 and 4). The concentration–response curves for these effects are bell-shaped, with inhibition seen at concentrations above 2 mM (Figs. 3 and 4). This presumably reflects an inhibitory action similar to that described above for intact cells. The activation of semi-permeabilized platelets by neomycin sulphate is inhibited completely by indomethacin (Table 1) and by 1 mM-EGTA (results not shown), but is not mimicked by K_2SO_4 (results not shown). With platelets prelabelled with [^{32}P]P_i or [^{3}H]inositol, neomycin (0.4 mM) stimulated formation of [^{32}P]phosphatidic acid and inositol [^{3}H]phosphates after permeabilization with saponin (Table 1); the formation of phosphatidic acid was inhibited completely by indomethacin (Table 1) (not studied for inositol phosphates).

A second aminoglycoside antibiotic, streptomycin, also stimulated aggregation and secretion responses in saponin-permeabilized platelets at relatively low concentrations (0.06–2 mM), and again inhibition was observed at higher concentrations (Figs. 3 and 4). Activation of semi-permeabilized platelets by streptomycin is inhibited completely by indomethacin (10 μM) and by EGTA (1 mM) (results not shown).

Concentrations of neomycin that were below threshold for secretion and aggregation responses (i.e. 0.006–0.02 mM) had no effect on the activation of saponin-permeabilized platelets by thrombin (results not shown).

DISCUSSION

The present study has shown that the aminoglycoside antibiotic neomycin inhibits aggregation of intact platelets by thrombin, collagen, ADP and A23187. Since, in the presence of indomethacin, A23187 [7] and ADP [6] do not activate platelets through inositol phospholipid hydrolysis, the inhibitory action of neomycin is unlikely to be related to an action on these lipids. This result is

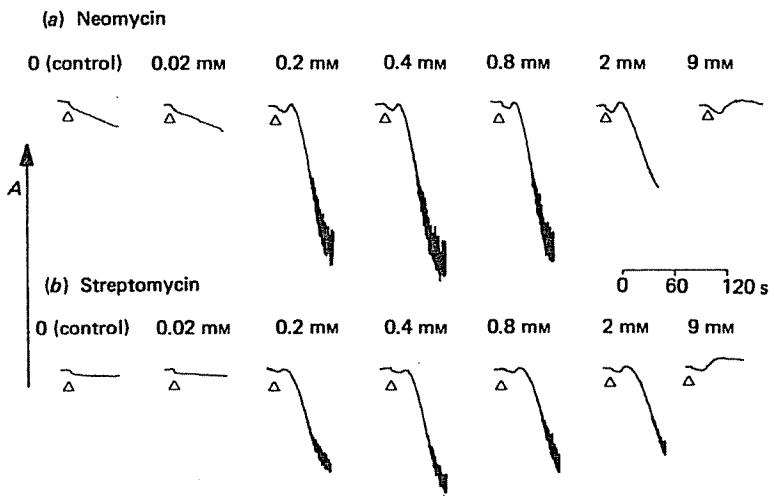

Fig. 3. Aminoglycosides induce aggregation of saponin-permeabilized human platelets

Washed platelets were incubated with neomycin or streptomycin for 1 min before addition of saponin (arrowhead) and left for a further 1 min. For description of traces see Fig. 2. The results are shown as typical traces from one experiment, which is representative of five others.

Table 1. Neomycin stimulates [^{3}H]5-hydroxytryptamine secretion and [^{32}P]phosphatidic acid and [^{3}H]inositol phosphate formation in semi-permeabilized platelets

Platelets were prelabelled with [^{32}P]P_i, [^{3}H]inositol or [^{3}H]5-hydroxytryptamine (5-HT) and challenged with saponin (17 μg/ml) alone or in the presence of neomcyin (0.4 mM) for 60 s. Release of [^{3}H]5-HT and formation of [^{32}P]phosphatidic acid (PA) or total [^{3}H]inositol phosphates (IPs) were measured as described in the Methods section. Results are shown as means ± S.E.M. for three or four experiments performed in triplicate or quadruplicate: $^*P < 0.05$ relative to saponin alone. Results for [^{3}H]5-HT secretion are given as percentage of total in tissue, and those for [^{32}P]PA and [^{3}H]IPs as d.p.m.

Parameter	Basal	Saponin	Saponin + neomycin	Saponin + neomycin + indomethacin
[^{3}H]5-HT release	0	23.0 ± 4.4	60.3 ± 10.0*	21.6 ± 3.9
[^{32}P]PA formation	696 ± 131	694 ± 72	1481 ± 191*	756 ± 96
[^{3}H]IPs formation	4804 ± 465	4873 ± 314	6493 ± 500*	–

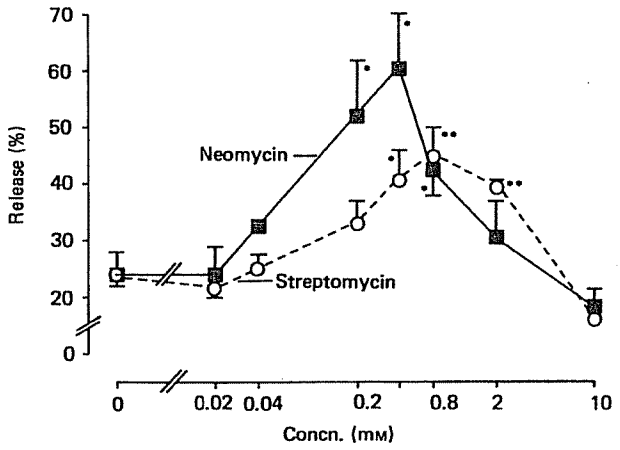

Fig. 4. Aminoglycosides stimulate 5-hydroxytryptamine secretion from saponin-permeabilized human platelets

Washed platelets, prelabelled with [^{3}H]5-hydroxytryptamine, were incubated with neomycin or streptomycin for 1 min before addition of saponin and left for a further 1 min. 5-Hydroxytryptamine secretion was estimated as described in the Methods section. Note that saponin causes a significant release of 5-hydroxytryptamine, as reported previously by ourselves [21] and others [22]. Results are means ± S.E.M. from four experiments: $^*P < 0.05$, $^{**}P < 0.01$ relative to saponin alone.

perhaps not surprising, since neomycin does not readily cross cell membranes, and the inositol phospholipids are predominantly located on the inner leaflet of the membrane. The concentration of neomycin required to inhibit aggregation [2–10 mM] is high enough that binding to other anionic phospholipids, e.g. phosphatidylserine and phosphatidic acid, is likely to occur [25], and this may account for its inhibitory action on aggregation. The selective inhibition of aggregation rather than of shape change indicates that neomycin may inhibit the cross-linking of platelets by fibrinogen or the binding of fibrinogen to its receptor. The selective inhibition of thrombin-induced shape change suggests a second site of action for neomycin in intact platelets; this site is unlikely to be related to the inositol phospholipids, since neomycin did not prevent shape change induced by collagen.

With semi-permeabilized platelets, relatively low concentrations of neomycin, and also streptomycin, stimulate secretion and aggregation, together with the formation of phosphatidic acid and inositol phosphates (not studied for streptomycin). All these effects are fully inhibited by indomethacin and EGTA. The rapid formation of phosphatidic acid [26] and inositol phosphates indicates activation of phospholipase C, and the sensitivity to indomethacin suggests that this occurs secondary to cyclo-oxygenase activation. The mechanism whereby neomycin activates phospholipase C is not known, but it is interesting that the present observations resemble those reported previously for activation of saponin-permeabilized platelets by inositol trisphosphate [21,22,27]. In this case it was hypothesized that the release of Ca^{2+} by inositol trisphosphate from intracellular stores led to the activation of phospholipase A_2. In this context, it may be worth considering that the lethal action of aminoglycosides on bacteria is associated with the leakage of small ions, an effect linked to the disruption of membrane structure [28]. Clearly, a direct study of the action of neomycin on Ca^{2+} mobilization from intracellular organelles is required.

The ability of neomycin to activate semi-permeabilized platelets at concentrations known to bind to polyphosphoinositides [8–16] is not easy to explain. There is no reason to suspect that neomycin will not bind to inositol phospholipids in platelet membranes, and yet activation by neomycin involves the hydrolysis of inositol phospholipids. Possible explanations which may account for this apparent paradox include compartmentalization within the semi-permeabilized platelets, or kinetic differences.

Since this paper was submitted, Siess & Lapetina [29] have reported that neomycin inhibits activation of intact platelets by thrombin, an observation that has been confirmed in the present study. They also reported that neomycin had no effect on platelet activation by other agonists, but in the present study we observed a significant inhibition of aggregation induced by collagen, ADP and A23187.

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Neomycin inhibits histamine and thapsigargin mediated Ca^{2+} entry in DDT_1 MF-2 cells independent of phospholipase C activation

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Abstract

The histamine H_1 receptor mediated increase in cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) was measured in the presence of the known phospholipase C (PLC) inhibitor, neomycin. Neomycin (1 mM) inhibited the histamine (100 μM) induced rise in $[Ca^{2+}]_i$ to the same extent as observed after blocking Ca^{2+} entry with $LaCl_3$. Likewise, the increase in $[Ca^{2+}]_i$ after re-addition of $CaCl_2$ (2 mM) to extracellular Ca^{2+} deprived and histamine pretreated cells was strongly reduced by neomycin. However, neomycin did not inhibit the histamine induced formation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) or the release of Ca^{2+} from internal stores. These results show that neomycin blocks histamine induced Ca^{2+} entry independent of phospholipase C activation. Inhibition of intracellular store Ca^{2+}-ATPase by thapsigargin (1 μM), elicited an increase in $[Ca^{2+}]_i$ due to a leakage from the stores, subsequently followed by store-dependent Ca^{2+} entry. Thapsigargin induced Ca^{2+} entry was also completely blocked by neomycin. These results indicate that neomycin inhibits histamine and thapsigargin induced Ca^{2+} entry. This inhibition is most likely exerted at the level of plasma membrane Ca^{2+} channels.

Keywords: Histamine H_1 receptor; Neomycin; Ca^{2+} entry; Phospholipase C

1. Introduction

Stimulation of plasma membrane receptors is often associated with an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), caused by the release of Ca^{2+} from internal stores and the entry of Ca^{2+} across the plasma membrane. It has been firmly established that agonist induced Ca^{2+} release is mediated by inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) (Streb et al., 1983; Berridge, 1993). Several mechanisms have been proposed to explain receptor mediated Ca^{2+} entry, including: opening of a ligand-gated Ca^{2+} channel (Benham and Tsien, 1987); activation of a Ca^{2+} channel by a GTP binding heterotrimeric protein (Matthews et al., 1989) and activation by a second messenger, such as $Ins(1,4,5)P_3$ (Kuno and Gardner, 1987; Restrepo et al., 1990; Mozhayeva et al., 1991), inositol 1,3,4,5-tetrakisphosphate ($Ins(1,3,4,5)P_4$, Morris et al., 1987; Lückhoff and Clapham, 1992) or arachidonic acid (Keyser and Alger, 1990; Van der Zee et al., 1995). Moreover, it was suggested that the filling state of intracellular Ca^{2+} stores determines the rate of Ca^{2+} entry (Putney, 1986). This pathway is activated by the Ca^{2+}-ATPase inhibitor, thapsigargin (Thastrup et al., 1990), causing an emptying of intracellular Ca^{2+} stores and possibly leading to the release of a cytosolic influx factor (Parekh et al., 1993; Randriamampita and Tsien, 1993; Thomas and Hanley, 1995). A physical link between luminal $Ins(1,4,5)P_3$ receptors and plasma membrane $Ins(1,3,4,5)P_4$ receptors was also suggested to activate Ca^{2+} entry (Irvine, 1992; Fadool and Ache, 1994).

In DDT_1 MF-2 smooth muscle cells, histamine H_1 receptor mediated $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ formation has been associated with Ca^{2+} release and Ca^{2+} entry (Molleman et al., 1991; Sipma et al., 1995a). Ca^{2+} entry was shown to be highly dependent on a continuous histamine H_1 receptor occupation, suggesting a strong store-independent component in histamine induced Ca^{2+} entry (Dickenson and Hill, 1992). In agreement, in a previous study based on patch-clamp measurements of Ca^{2+} activated K^+ currents, we showed that histamine evoked Ca^{2+}

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entry can occur in the absence of Ca^{2+} release and is therefore not completely dependent on the emptying of internal Ca^{2+} stores (Van der Zee et al., 1995). It was suggested that arachidonic acid is involved in histamine induced Ca^{2+} entry (Van der Zee et al., 1995). The aim of this study was to investigate whether phospholipase C activation is required for generating a messenger involved in the regulation of Ca^{2+} entry. The antibiotic and anti-arrhythmic drug neomycin (Anderson et al., 1995; Woodcock, 1995) is known to bind to phosphatydylinositol(4,5)bisphosphate, thereby inhibiting phospholipase C activation (Orsulakova et al., 1976; Schacht, 1976) and Ins(1,4,5)P_3 formation (Carney et al., 1985). Therefore, we determined the modulation of histamine H_1 receptor mediated and store-dependent Ca^{2+} entry by neomycin.

2. Materials and methods

2.1. Cell culture

DDT_1 MF-2 smooth muscle cells, derived from a Syrian hamster vas deferens were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 7 mM $NaHCO_3$, 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (Hepes, pH 7.2) and 10% fetal calf serum at 37°C in 5% CO_2.

2.2. Measurement of Ins(1,4,5)P_3

DDT_1 MF-2 cells were grown in monolayers in 9.6 cm^2 plastic wells and an experimental protocol described earlier (Sipma et al., 1995b) was followed. The medium was replaced by 2 ml DMEM at 20°C, 30 min before starting the experiment by adding agonists. After removing the medium, reactions were stopped with 400 μM 5% trichloroacetic acid and placed on ice for at least 45 min. Samples were washed 3 times with 800 μl water saturated diethylether and neutralised with KOH (25 μl, 0.2 M).

Mass measurements of Ins(1,4,5)P_3 were performed as described earlier, using a standard curve of Ins(1,4,5)P_3 in ether extracted trichloroacetic acid-solution (Molleman et al., 1991). In brief, samples were assayed in 25 mM Tris/HCl (pH 9.0), 1 mM EDTA, 1 mg bovine serum albumin, [^{3}H]Ins(1,4,5)P_3 (3,3 Ci/mmol, 2000 cpm/assay) and about 1 mg binding protein for 15 min. The binding protein was isolated from fresh beef liver (Chilvers et al., 1989). Bound and free radioactivities were separated by centrifugation. The radioactivity in the pellet was determined by liquid scintillation counting.

2.3. Measurements of intracellular Ca^{2+}

$[Ca^{2+}]_i$ was measured by Fura-2 fluorescence. Individual glass coverslips covered with a monolayer of DDT_1 MF-2 cells were placed in 10 cm^2 plastic petri dishes and 2 ml buffered salt solution (BBS) containing NaCl (145 mM), KCl (5 mM), $MgSO_4$ (0.5 mM), $CaCl_2$ (1 mM), D-glucose (10 mM), Hepes (10 mM, adjusted to pH 7.4) were added. Fura-2 was loaded in the cytosol by incubation with Fura-2/AM (3 μM) for 45 min at 37°C in BBS supplemented with 1% bovine serum albumin. The coverslip with cells was washed quickly 3 times by placing it in fresh BBS and left in BBS (22°C) for 10 min. Thereafter the coverslip was mounted in a specially designed holder and placed in a quartz cuvette. Total volume (BBS) in the cuvette was 2 ml and agonists and inhibitors were added in 20 μl portions without opening the cuvette chamber. Measurements were performed at 22°C. Under Ca^{2+} free conditions the cells were washed and Fura-2 fluorescence was measured in BBS without $CaCl_2$ but supplemented with 0.1 mM EGTA. Excitation wavelengths were 340 nm and 380 nm and the emission wavelength was 510 nm. The ratios of emitted light at 510 nm was acquired every 1.0 s. These ratios were converted to Ca^{2+} levels using the classical equation described by Grynkiewicz et al. (1985). The R_{max} of the equation was measured in the presence of 2.0 mM $CaCl_2$ and 10 μM ionomycin. The R_{min} was measured in the presence of 10 μM ionomycin and 50 mM EGTA (adjusted to pH 8). The autofluorescence of the cell was determined as fluorescence remaining in the presence of 5 mM $MnCl_2$ and 10 μM ionomycin.

2.4. ^{3}[H]Arachidonic acid release

Arachidonic acid release was measured as described previously (Van der Zee et al., 1995). In brief, cells grown in 6-well plastic petri dishes (Costar) were labelled with 0.5 μCi ^{3}[H]arachidonic acid/10^6 cells/well in serum free culture medium (1 ml) for 3 h at 37°C. To eliminate unincorporated activity, cells were washed with buffered salt solution (BSS) containing: NaCl (145 mM), KCl (5 mM), $CaCl_2$ (1.4 mM), $MgSO_4$ (0.5 mM), glucose (10 mM), Hepes (10 mM, adjusted to pH 7.4), twice with BSS supplemented with 1% bovine serum albumin (essentially fatty acid free) and once again with BSS before equilibration for 25 min at 22°C. Complete washing was performed within 45 s. Cells were pretreated with neomycin (1 mM) during the equilibration period and during 20 min before the washing procedure. After this, cells were exposed to the indicated agonists, the solution was collected at the indicated time-points and ^{3}[H]arachidonic acid release was determined by liquid scintillation counting.

2.5. Data analysis

Data are represented as means ± S.E.M. Values were considered significantly different from control when $P < 0.05$ as determined using Student's unpaired t-test. A sigma plot logistic curve fit program (Jandel Scientific, USA) was used to determine EC_{50} values and to analyze

binding parameters obtained from the Ins(1,4,5)P_3 radioligand binding assay.

2.6. Chemicals

Fura-2/AM and Ins(1,4,5)P_3 sodium salt were obtained from Boehringer (Germany). Thapsigargin, neomycin sulphate and bovine serum albumin (essentially fatty acid free) were purchased from Sigma (USA). Histamine dihydrochloride was from Fluka (Switzerland). D-[2-^{3}H]Inositol 1,4,5-trisphosphate and [^{3}H]arachidonic acid were from Du Pont-New England Nuclear (USA). Lanthanum chloride, Hepes and all other chemicals were from Merck (Germany).

3. Results

The histamine H_1 receptor mediated increase in $[Ca^{2+}]_i$ was measured in the presence of neomycin. Histamine (100 μM) evoked a rapid increase in $[Ca^{2+}]_i$, reaching a maximum after about 30 s, which was mainly caused by Ca^{2+} release from internal stores. This initial rise in $[Ca^{2+}]_i$ was followed by a slowly declining phase, due to Ca^{2+} entry from the extracellular space (Fig. 1A, Table 1). Pretreatment of cells with neomycin (1 mM, 45 min) slightly reduced the initial rise in $[Ca^{2+}]_i$ and abolished the slowly declining component of the histamine induced response (Fig. 1B, Table 1). Similar results were obtained after blocking Ca^{2+} entry with $LaCl_3$ (Table 1), known to act directly on plasma membrane Ca^{2+} channels (Den Hertog et al., 1992).

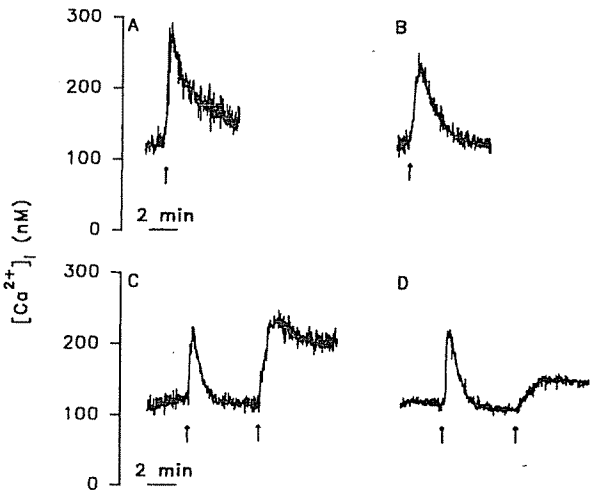

Fig. 1. The effect of neomycin on histamine H_1 receptor mediated changes in $[Ca^{2+}]_i$. The histamine (100 μM) induced increase in $[Ca^{2+}]_i$ was measured in the presence of extracellular Ca^{2+} in (A) non-pretreated cells and (B) in cells pretreated with neomycin (1 mM, 45 min). The arrow indicates the addition of histamine. The histamine (100 μM, first arrow) induced increase in $[Ca^{2+}]_i$ was also measured in the absence of extracellular Ca^{2+} in (C) non-pretreated cells and (D) in cells pretreated with neomycin (1 mM, 45 min). Extracellular Ca^{2+} (2 mM) was added after 5 min of stimulation of the cells with histamine (second arrow). Each tracing represents a typical result out of at least 6 experiments.

Table 1
Effect of neomycin or $LaCl_3$ on the histamine or thapsigargin induced increase in $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} in DDT$_1$ MF-2 cells

	Increase in $[Ca^{2+}]_i$ (nM)	
	Maximal increase	Sustained phase
Treatment		
Histamine	160±16	57±9
Neomycin + histamine	122±12 [a]	10±5 [a]
$LaCl_3$ + histamine	115±9 [a]	3±2 [a]
Thapsigargin	130±12	26±5
Neomycin + thapsigargin	43±3 [a]	0±0 [a]
$LaCl_3$ + thapsigargin	41±5 [a]	0±0 [a]

The histamine (100 μM) or thapsigargin (1 μM) induced increase in $[Ca^{2+}]_i$ was measured in non-pretreated cells and in cells pretreated with neomycin (1 mM, 45 min) or $LaCl_3$ (50 μM, 2 min). The sustained increase in $[Ca^{2+}]_i$ was measured 2 min after the addition of histamine or 10 min after the addition of thapsigargin. Basal $[Ca^{2+}]_i$ values; non-pretreated: 130±4 nM; neomycin: 120±6 nM; $LaCl_3$: 126±6 nM. [a] Different from value obtained from non-pretreated cells, $P < 0.01$. Data are expressed as means±S.E.M. of at least 6 experiments.

In the absence of extracellular Ca^{2+}, histamine (100 μM) elicited a transient rise in $[Ca^{2+}]_i$ (Fig. 1C, Table 2), as observed in the presence of $LaCl_3$. This increase in $[Ca^{2+}]_i$, which is due to the release of Ca^{2+} from Ins(1,4,5)P_3 sensitive internal stores, was not affected by pretreatment of cells with neomycin (Fig. 1D, Table 2). In agreement, the maximal histamine induced Ins(1,4,5)P_3 formation, measured after 1 min of stimulation of cells (Sipma et al., 1995a) was not affected by neomycin (1 mM, Fig. 2). Re-addition of extracellular Ca^{2+} (2 mM), 5

Table 2
Effect of neomycin on histamine or thapsigargin induced Ca^{2+} release and Ca^{2+} entry in DDT$_1$ MF-2 cells

	Increase in $[Ca^{2+}]_i$ (nM)	
	Absence of Ca^{2+}	2 mM Ca^{2+}
Treatment		
None		73±9
Neomycin		43±2 [a]
$LaCl_3$		38±3 [a]
Histamine	113±8	138±10
Neomycin + histamine	105±8	51±6 [a]
Thapsigargin	41±3	203±21
Neomycin + thapsigargin	46±4	53±8 [a]

The histamine (100 μM) or thapsigargin (1 μM) induced increase in $[Ca^{2+}]_i$ were measured in the absence of extracellular Ca^{2+} (Ca^{2+} release) in non-pretreated cells and in cells pretreated with neomycin (1 mM, 45 min) or $LaCl_3$ (50 μM, 2 min). Ca^{2+} entry was measured as maximal increase in $[Ca^{2+}]_i$ after the addition of 2 mM Ca^{2+} to the solution 5 min after challenge of the cells with histamine or thapsigargin. Basal $[Ca^{2+}]_i$ values in the absence of extracellular Ca^{2+}; non-pretreated: 113±4 nM; neomycin: 111±4 nM. [a] Different from value obtained from non-pretreated cells, $P < 0.01$. Data are expressed as means±S.E.M. of at least 6 experiments.

min after the challenge of cells with histamine, gave rise to an initial rapid increase in $[Ca^{2+}]_i$ (overshoot), followed by a maintained elevated level after about 2 min (Fig. 1C, Table 2). Neomycin inhibited this rise in $[Ca^{2+}]_i$ induced by the re-addition of extracellular Ca^{2+} (Fig. 1D, Table 2). It was observed that in the absence of histamine, the addition of extracellular Ca^{2+} also elicited a substantial increase in $[Ca^{2+}]_i$ (Table 2), without the transient overshoot (not shown). This unstimulated rise in $[Ca^{2+}]_i$ observed on the addition of extracellular Ca^{2+} to neomycin pretreated cells was similar as that measured after blocking Ca^{2+} channels with $LaCl_3$ (Table 2).

Besides Ca^{2+} entry that is dependent on occupation of histamine H_1 receptors (Dickenson and Hill, 1992; Van der Zee et al., 1995), histamine may also provoke Ca^{2+} entry regulated by the filling state of internal Ca^{2+} stores (Putney, 1986). Inhibition of intracellular Ca^{2+}-ATPase pumps by thapsigargin (1 μM, Thastrup et al., 1990) caused a slowly evolving and sustained increase in $[Ca^{2+}]_i$ (Fig. 3A, Table 1). In the absence of extracellular Ca^{2+}, the Ca^{2+} response was transient and much smaller and re-addition of extracellular Ca^{2+} caused a strong and rapid increase in $[Ca^{2+}]_i$ (Fig. 3C, Table 2). Reduction of the thapsigargin induced rise in $[Ca^{2+}]_i$ was also observed in the presence of $LaCl_3$ (50 μM). Moreover, when $LaCl_3$ was added 5 min after thapsigargin, $[Ca^{2+}]_i$ rapidly declined to the basal unstimulated level (not shown). These results suggest store-dependent Ca^{2+} entry in DDT_1 MF-2 cells. Since arachidonic acid generation after stimulation with histamine is partly responsible for Ca^{2+} entry in DDT_1 MF-2 cells (Van der Zee et al., 1995), we determined the formation of arachidonic acid after stimulation with thapsigargin. Thapsigargin failed to induce arachidonic acid formation (basal: 318 ± 12 dps/10^6 cells; thapsigargin 1 μM, 5 min: 327 ± 25 dps/10^6 cells; histamine 100 μM, 15 s: 429 ± 11 dps/10^6 cells*, $P < 0.05$, $n = 8$). Neomycin strongly inhibited both the thapsigargin evoked

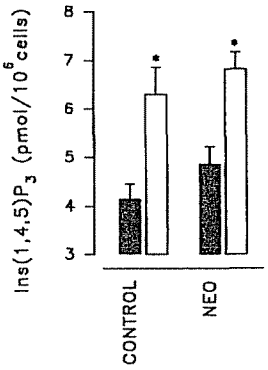

Fig. 2. Histamine H_1 receptor mediated formation of $Ins(1,4,5)P_3$. Basal $Ins(1,4,5)P_3$ (solid bars) and the histamine (100 μM, 60 s) induced formation of $Ins(1,4,5)P_3$ (open bars) was measured in non-pretreated cells (CONTROL) and in cells pretreated with neomycin (NEO, 1 mM, 45 min). * Different from the respective unstimulated value, $P < 0.05$. Data are expressed as means ± S.E.M. of 6 experiments.

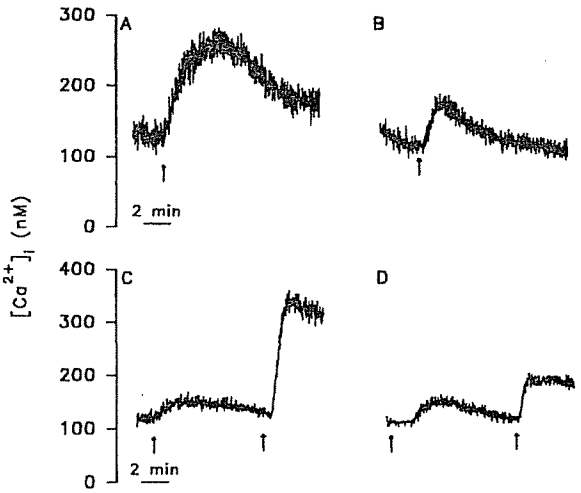

Fig. 3. The effect of neomycin on thapsigargin induced changes in $[Ca^{2+}]_i$. The thapsigargin (1 μM) induced rise in $[Ca^{2+}]_i$ was measured in the presence of extracellular Ca^{2+} in (A) non-pretreated cells and (B) in cells pretreated with neomycin (1 mM, 45 min). The arrow indicates the addition of thapsigargin. The thapsigargin (1 μM, first arrow) induced increase in $[Ca^{2+}]_i$ was also measured in the absence of extracellular Ca^{2+} in (C) non-pretreated cells and (D) in cells pretreated with neomycin (1 mM, 45 min). Extracellular Ca^{2+} (2 mM) was added after 5 min of stimulation of the cells with thapsigargin (second arrow). Each tracing represents a typical result out of at least 6 experiments.

rise in $[Ca^{2+}]_i$ when extracellular Ca^{2+} was available (Fig. 3B, Table 1) and after the addition of Ca^{2+} to the Ca^{2+} free solution (Fig. 3D, Table 2). The response to thapsigargin in the absence of extracellular Ca^{2+} (Fig. 3C) was not affected by neomycin (Fig. 3D, Table 2). The Ca^{2+} channels activated by both histamine (see also Dickenson and Hill, 1992) or thapsigargin are not permeable to Mn^{2+}, since the quenching-rate of Fura-2, induced by basal Mn^{2+} entry (Hallam and Rink, 1985) was not increased by histamine or thapsigargin (not shown).

4. Discussion

In this study, we investigated the involvement of phospholipase C activity to generate a messenger regulating Ca^{2+} entry in DDT_1 MF-2 cells. It is shown that histamine H_1 receptor mediated Ca^{2+} entry was completely abolished in the presence of neomycin and $LaCl_3$. However, this action on Ca^{2+} entry of neomycin is independent on phospholipase C activity, reflected by the unaffected histamine induced $Ins(1,4,5)P_3$ formation in DDT_1 MF-2 cells.

Since histamine evokes Ca^{2+} release from internal stores, it is supposed to cause Ca^{2+} entry that is dependent on the filling state of the store (Putney, 1986). Store-dependent Ca^{2+} entry induced by thapsigargin that inhibits the intracellular store Ca^{2+} ATPase (Thastrup et al., 1990; Bian et al., 1991) was also completely inhibited by

neomycin. Basal Ca^{2+} entry, measured after addition of $CaCl_2$ to extracellular Ca^{2+}-deprived cells is also inhibited by neomycin and $LaCl_3$. This basal Ca^{2+} entry might be caused by the 'leaking out' of cytoplasmic Ca^{2+}, resulting in a reduced filling-state of intracellular stores, consequently provoking store-dependent Ca^{2+} entry. Remarkably, Dickenson and Hill (1992) did not detect an increase in $[Ca^{2+}]_i$ upon the addition of extracellular Ca^{2+} to unstimulated and extracellular Ca^{2+}-deprived cells at 37°C. In contrast, a marked increase in $[Ca^{2+}]_i$ was observed under our experimental conditions (22°C). Since intracellular store-Ca^{2+} ATPase pumps are more activated at 37°C than at 22°C (Squier et al., 1988; Kalabokis and Hardwicke, 1988), apparently the rates of Ca^{2+} entry and uptake equalize at 37°C, leading to a no-net increase in $[Ca^{2+}]_i$. The Ca^{2+} entry remaining in the presence of neomycin and $LaCl_3$ (Table 2) most likely reflects the transition from the basal $[Ca^{2+}]_i$ in the absence to that in the presence of extracellular Ca^{2+} and might be mediated by non-specific cation channels or Na^+/Ca^{2+} exchange. These mechanisms were not activated by histamine or thapsigargin.

Direct histamine H_1 receptor-dependent Ca^{2+} entry and the indirect store-dependent mechanism stimulated by thapsigargin (and histamine) were inhibited by neomycin. Neomycin most likely inhibits Ca^{2+} entry by blocking the plasma membrane Ca^{2+} channels, as suggested also for hepatocytes (Altin and Bygrave, 1987). Therefore, neomycin is not a suitable tool to study the effects of messengers generated downstream of phospholipase C activation on receptor mediated and capacitive Ca^{2+} entry in DDT_1 MF-2 cells. Interestingly, in ischemic hearts, the inositol phosphate formation induced by reperfusion was strongly reduced by neomycin as well as by removing Ca^{2+} from the reperfusion solution (Anderson et al., 1995). In accordance with our results, we suggest that neomycin may act as a anti-arrhithmic drug (Woodcock, 1995) by preventing Ca^{2+} entry, rather than by a direct inhibition of $Ins(1,4,5)P_3$ formation.

In a previous study we reported that histamine induced Ca^{2+} entry still occurred if the Ca^{2+} release process and the concomitant store-dependent Ca^{2+} entry was inhibited (Van der Zee et al., 1995). Histamine induced arachidonic acid formation was supposed to be in involved in histamine H_1 receptor mediated Ca^{2+} entry. Moreover, histamine induced Ca^{2+} entry was shown to be dependent on histamine H_1 receptor occupation in DDT_1 MF-2 cells (Dickenson and Hill, 1992). Store-dependent Ca^{2+} entry however, as activated by thapsigargin, is not mediated by arachidonic acid, because thapsigargin does not induce arachidonic acid formation.

In conclusion, neomycin inhibits the plasma membrane Ca^{2+} channels that can be activated by a histamine H_1 receptor-dependent pathway and the channels activated by a mechanism that is dependent on the filling-state of intracellular Ca^{2+} stores. The inhibitory action of neomycin on histamine induced Ca^{2+} entry is not dependent on phospholipase C activity.

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EXHIBIT D

Polarity of TRH receptors in transfected MDCK cells is independent of endocytosis signals and G protein coupling

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Yeaman, Charles, Marcos Heinflink, Erik Falck-Pedersen, Enrique Rodriguez-Boulan, and Marvin C. Gershengorn. Polarity of TRH receptors in transfected MDCK cells is independent of endocytosis signals and G protein coupling. *Am. J. Physiol.* 270 (*Cell Physiol.* 39): C753–C762, 1996.—Information concerning the molecular sorting of G protein-coupled receptors in polarized epithelial cells is limited. Therefore, we have expressed the receptor for thyrotropin-releasing hormone (TRH) in Madin-Darby canine kidney (MDCK) cells by adenovirus-mediated gene transfer to determine its distribution in a model cell system and to begin analyzing the molecular information responsible for its distribution. Equilibrium binding of [*methyl*-^{3}H]TRH to apical and basolateral surfaces of polarized MDCK cells reveals that TRH receptors are expressed predominantly (>80%) on the basolateral cell surface. Receptors undergo rapid endocytosis following agonist binding; up to 80% are internalized in 15 min. A mutant receptor missing the last 59 residues, C335Stop, is poorly internalized (<10%) but is nevertheless basolaterally expressed (>85%). A second mutant TRH receptor, Δ218–263, lacks essentially all of the third intracellular loop and is not coupled to G proteins on binding agonist. This receptor internalizes TRH approximately half as efficiently as wild-type TRH receptors but is nevertheless strongly polarized to the basolateral surface (>90%). These results indicate that molecular sequences responsible for basolateral accumulation of TRH receptors can be segregated from signals for ligand-induced receptor endocytosis and coupling to heterotrimeric G proteins.

basolateral sorting; Madin-Darby canine kidney cells; replication-defective adenovirus; thyrotropin-releasing hormone receptor

THE ESTABLISHMENT OF specialized apical and basolateral cell surfaces of polarized epithelial cells involves the selective delivery of newly synthesized membrane proteins to correct destinations and the retention of appropriate proteins at these sites (reviewed in Refs. 19 and 20). The most thoroughly investigated model for epithelial polarity is the Madin-Darby canine kidney (MDCK) cell line. In these cells, the sorting of apical and basolateral proteins from each other occurs in the *trans*-Golgi network (TGN). Molecular sorting of membrane proteins in the TGN requires discrete structural motifs in cargo molecules specifying transport to either the apical or basolateral plasma membrane domain. For example, the glycosylphosphoinositide anchor of certain proteins serves to target these molecules to the apical plasma membrane (16). Short cytoplasmic amino acid sequences that function as dominant basolateral sorting signals are being identified in a growing number of integral membrane proteins that span the lipid bilayer a single time (reviewed in Ref. 19). Many of these basolateral sorting signals are similar to tyrosine- or dileucine-based endocytic motifs and frequently serve in this capacity or, as shown for TGN 38/41, lgp120, and lysosomal acid phosphatase, as TGN or lysosomal localization motifs (11, 26, 27).

Not surprisingly, very little is known concerning the molecular determinants directing polarized sorting of complex polytypic membrane proteins in epithelial cells. At a minimum, the presence of multiple cytoplasmic, transmembrane, and extracellular domains in these proteins suggests the possibility of distinct types of hierarchical and competing targeting signals and makes the task of identifying the sorting mechanisms responsible for the transport of these molecules from the TGN to the cell surface particularly daunting. The polarized trafficking of only one G protein-coupled receptor, namely the α_{2A}-adrenergic receptor, has been carefully studied. In stably transfected MDCK cells, this receptor is targeted directly to the basolateral membrane (13). Efforts to identify discrete basolateral targeting determinants in this protein were negative, and it was speculated that sorting of α_{2A}-adrenergic receptors is mediated either by structural information residing within the membrane-spanning segments or by the tertiary structure of the molecule (12).

The receptor for thyrotropin-releasing hormone (TRH) belongs to the rhodopsin family of seven transmembrane-spanning G protein-coupled receptors (Fig. 1) (29). TRH stimulates the secretion of thyroid-stimulating hormone and prolactin from the anterior pituitary gland and acts as a neurotransmitter and/or neuromodulator in the nervous system. The actions of TRH as a pituitary hormone secretagogue have been studied extensively (6). Agonist occupation of the TRH receptor leads to activation of a heterotrimeric guanine nucleotide-binding regulatory protein ($G\alpha_{11}$ or $G\alpha_q$) that, in turn, activates an effector enzyme, phosphatidylinositol-specific phospholipase C-β. Subsequent hydrolysis of phosphatidylinositol-4,5-bisphosphate generates two intracellular second messengers, inositol 1,4,5-trisphosphate and 1,2-diacylglycerol, leading to activation of protein kinase C and increased intracellular calcium. The biology of the TRH receptor suggests that it should be concentrated in membranes exposed to TRH in the bloodstream and nervous system, such as the basolateral membrane of pituitary epithelial cells and the somatodendritic membrane of postsynaptic neurons, respectively.

In the present study, we have expressed the TRH receptor in polarized MDCK cells using a replication-

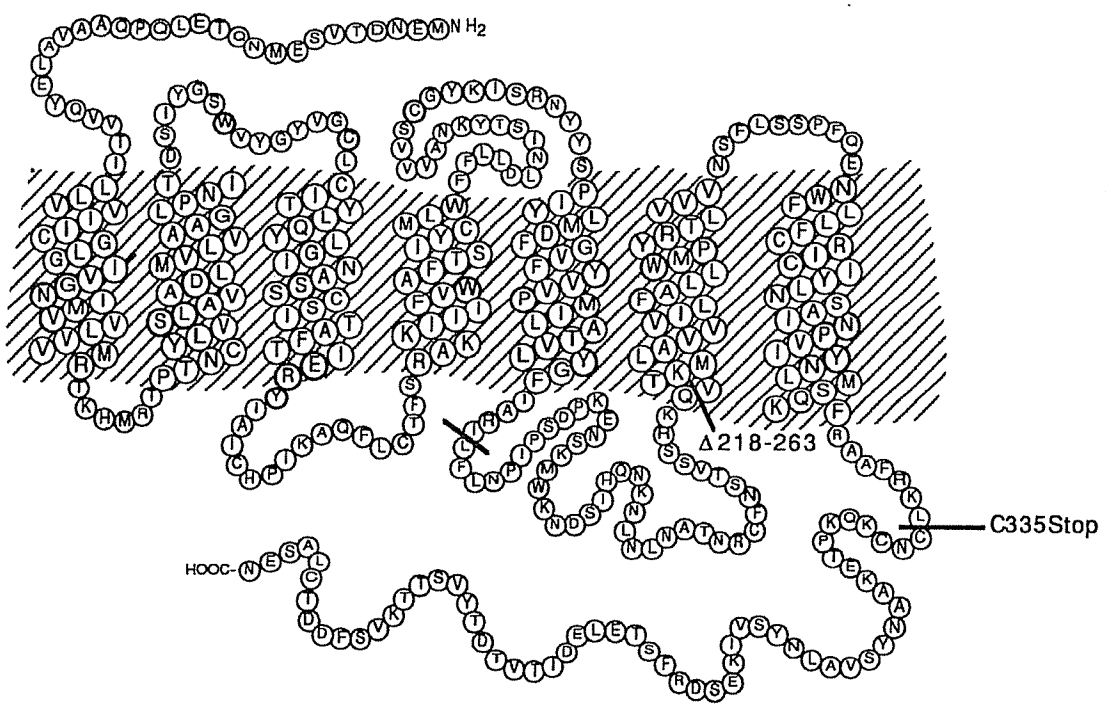


Fig. 1. Schematic representation of murine thyrotropin-releasing hormone (TRH) receptor and deletion mutants evaluated in present study. Topographical model indicates 7 transmembrane-spanning segments, with extracellular domains above and cytoplasmic domains below plasma membrane (hatched area). Cytoplasmic carboxy-terminal domain shows position of stop codon inserted to generate mutant C335Stop TRH receptors (solid bar). Boundaries of third intracellular loop amino acid sequence deleted in Δ218–263 TRH receptors are also indicated.

defective adenovirus vector to study its distribution and to investigate the relationship among signals mediating sorting, endocytosis, and G protein coupling of this receptor. Wild-type TRH receptors are expressed predominantly on the basolateral surface of MDCK cells, where they stimulate inositol phosphate (IP) formation and undergo rapid endocytosis following agonist binding. Mutant TRH receptors lacking the carboxy-terminal cytoplasmic domain or the large third intracellular loop are severely compromised in endocytic and signaling functions, respectively, but are nonetheless expressed almost exclusively on the basolateral surface. These studies thus indicate that molecular information specifying basolateral targeting of this G protein-coupled receptor can be segregated from signals for ligand-induced receptor endocytosis and coupling to heterotrimeric G proteins.

MATERIALS AND METHODS

Materials. High-glucose Dulbecco's modified Eagle's medium (DMEM) and [3-*methyl*-His^2]TRH (MeTRH) were purchased from Sigma (St. Louis, MO); Hanks' balanced salt solution and MEM nonessential amino acids were from GIBCO BRL (Grand Island, NY); fetal bovine serum was from Gemini Bioproducts (Calabasas, CA); [*methyl*-^{3}H]TRH (40 Ci/mmol) was from Du Pont-New England Nuclear (Boston, MA); and *myo*-[^{3}H]inositol was from Amersham (Arlington Heights, IL). Monoclonal antibody (MAb) against uvomorulin was a generous gift from Dr. B. Gumbiner (9) and MAb against gp135 was generously provided by Dr. G. Ojakian (23).

Cell culture and adenovirus infections. MDCK type II cells were grown in DMEM supplemented with 5% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2. For polarity experiments, cells were seeded at a density of 2×10^6 cells per 24.5-mm polycarbonate membrane filter (Transwells, 0.4 μm pore size, Corning-Costar, Cambridge, MA) and cultured for 4–6 days with changes of medium every other day. Transepithelial resistance was measured using a Millipore device (Millipore, Bedford, MA) and was routinely 200–300 $\Omega \cdot cm^2$.

The construction of the adenovirus-based expression vector AdCMVmTRHR has been described previously (4). The virus AdCMVmTRHR-C335Stop was constructed in an identical fashion. This replication-defective virus contains the coding sequence of a truncated mutant (C335Stop) mouse TRH receptor lacking the last 59 amino acids (21). For infection, cultures were washed once with serum-free DMEM, and AdCMVmTRHR [1–100 plaque-forming units (PFU) or 30–3,000 particles per cell] was added to the apical culture chamber. Adenovirus infection is roughly 50-fold more efficient from the apical surface than from the basolateral surface of MDCK cells (Yeaman and Rodriguez-Boulan, unpublished observations). Cultures were incubated with virus at 37°C for 1 h before an equal volume of DMEM containing 10% fetal bovine serum was added to both apical and basolateral chambers and the incubation was continued for 6–48 h. During binding assays (see below), the integrity of monolayers was confirmed by monitoring the leakage of [*methyl*-^{3}H]TRH from the apical compartment to the basolateral compartment and vice versa by sampling radioactivity in both compartments. In all cases, leakage of ligand across the filters was <2% per hour.

Construction of the plasmid encoding the mutant Δ218–263 TRH receptor has been described previously (22). MDCK II cells were stably transfected by electroporation with plasmids encoding either wild-type or Δ218–263 TRH receptors and pRSV/Neo. Stable transfectants were selected with G418

(400 μg/ml active), and clones were screened for receptor expression by radiolabeled ligand binding (see below).

Measurement of TRH receptor number. The number of TRH receptors expressed at the apical and basolateral cell surface was measured by radioligand binding (5). Briefly, 0.1–10.0 nM [*methyl*-^{3}H]TRH, an analogue of higher affinity than TRH, was applied in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer exclusively to either the apical or basolateral culture compartment. Surfaces not receiving ligand were incubated in HEPES buffer alone. To quantify nonspecific binding, incubations were performed in the presence of 1 μM unlabeled MeTRH. Incubations were performed at 37°C for 5–90 min or at 0°C for 4 h. After incubation with ligand, filters were washed extensively with ice-cold buffer, excised from the chamber, and lysed in 0.4 N NaOH. The lysate and filter were transferred to vials, mixed with scintillation cocktail, and analyzed by scintillation counting. Binding isotherms were fitted, and equilibrium dissociation constants (K_d values) and receptor numbers were obtained with the INPLOT program (GraphPAD). The data are calculated with the assumption of a one-to-one stoichiometry of ligand to receptor and a homogeneous distribution of TRH receptors in all cells in the population.

Internalization of TRH receptors was measured as specifically bound [*methyl*-^{3}H]TRH that was resistant to acid wash (10, 21). Specific, acid-resistant binding was calculated by subtracting the nonspecific from the [*methyl*-^{3}H]TRH remaining after incubation at 4°C for 2 min with 0.2 M acetic acid and 0.5 M NaCl, pH 2.5, applied to both apical and basolateral cell surfaces.

Measurement of TRH-stimulated IP formation. Cultures were infected with 10–20 PFU/cell AdCMVmTRHR and prelabeled for 24–48 h with 1 μCi/ml *myo*-[^{3}H]inositol before the magnitude of TRH-stimulated IP formation was measured. Filter-grown cells were stimulated from the apical, basolateral, or both surfaces for 2.5–60 min at 37°C with 0–1,000 nM MeTRH. Stimulation by MeTRH was performed at 37°C in growth medium containing 10 mM LiCl and *myo*-[^{3}H]inositol, to prevent depletion of ^{3}H-labeled phosphoinositide substrate. After stimulation, filters were excised, phospholipids were extracted in chloroform/methanol/HCl, and phase separation was performed. [^{3}H]IPs were quantified as described (7).

Cell surface biotinylation. Biotinylation of confluent monolayers on Transwells with sulfo-*N*-hydroxy-succinimidobiotin (Pierce) was carried out twice for 20 min at 4°C as described (17). Cell lysates were immunoprecipitated as described (14) with the use of a MAb against uvomorulin (9) (diluted 1/200) or a MAb against gp135 (23) (diluted 1/200). After immunoprecipitation, samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Biotinylated proteins were then revealed by blotting with ^{125}I-streptavidin (17).

Statistical analysis. All experiments were performed at least twice. The individual data points represent duplicate or triplicate determinations in a single experiment. Statistical significance was determined by *t*-test.

RESULTS

Expression of functional TRH receptors in MDCK cells following adenovirus-mediated gene transfer. The overall objectives of this study were to characterize the distribution of TRH receptors in polarized MDCK cells and to begin analyzing the molecular information controlling this distribution. However, these cells do not express TRH receptors endogenously (Fig. 2*A*). Therefore, the cDNA encoding this protein was introduced into MDCK cells by adenovirus-mediated gene transfer, as previously reported for a variety of nonepithelial cell types (4, 7). To determine whether MDCK cells can be infected by recombinant human adenoviruses, nonpolarized subconfluent cultures were incubated with increasing numbers of AdCMVmTRHR and specific binding of [*methyl*-^{3}H]TRH was quantified 24 h later. This analysis revealed that increasing numbers of cell surface TRH receptors are expressed by cultures infected with increasing numbers of viruses (Fig. 2*A*). With infection by 100 PFU AdCMVmTRHR per cell, there were ~7 × 10^5 receptors per MDCK cell, assuming that all cells express equal numbers of TRH receptors. Infection at multiplicities between 10 and 30 consistently results in ~5–20 × 10^4 sites/cell, an expression level comparable with that of endogenous TRH receptors in clonal rat pituitary GH cells (6).

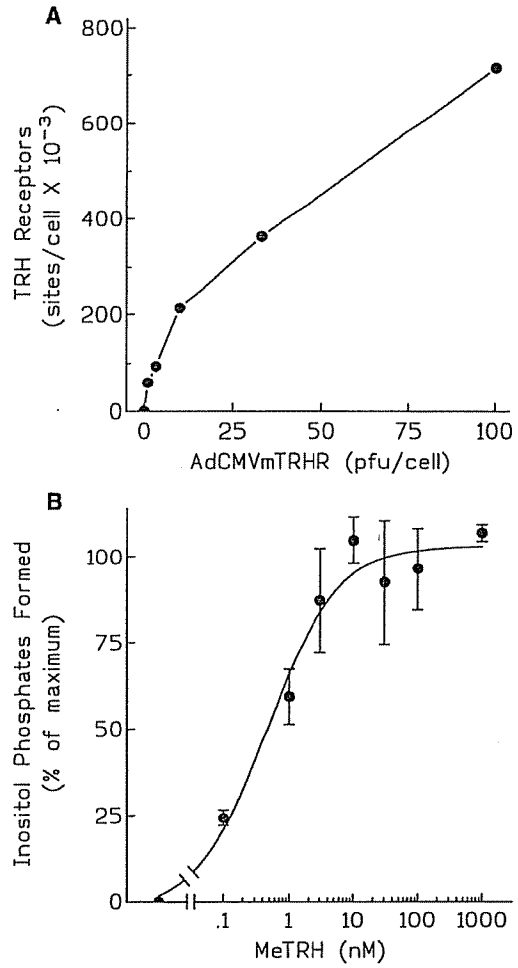


Fig. 2. Expression of TRH receptors and stimulation of inositol phosphate (IP) formation in Madin-Darby canine kidney (MDCK) cells infected by AdCMVmTRHR. *A*: monolayers of MDCK cells cultured on plastic substrata were infected with AdCMVmTRHR at indicated multiplicities of infection. After 24 h, numbers of TRH receptors were measured as described under MATERIALS AND METHODS. *B*: MDCK cells, cultured as in *A*, were infected with 10 plaque-forming units (PFU)/cell AdCMVmTRHR and prelabeled with *myo*-[^{3}H]inositol. After 24 h, [3-*methyl*-His^2]TRH (MeTRH) stimulation of IP formation in cells was measured as described under MATERIALS AND METHODS. Data points and error bars represent means ± SD of triplicate determinations in a representative experiment.

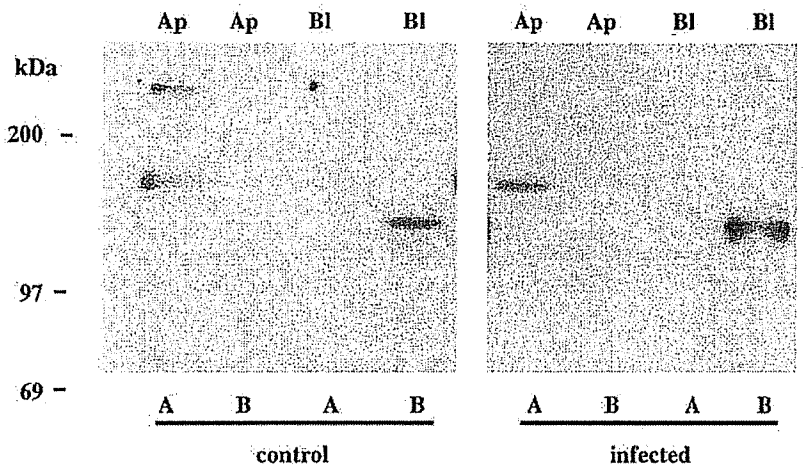


Fig. 3. Polarized distribution of apical gp135 and basolateral uvomorulin on MDCK cells infected by a recombinant adenovirus. Polarized MDCK cells on Transwells were mock infected (control) or infected with 50 PFU/cell AdCMV-CAT (infected); 24-h later, cells were biotinylated either from apical (Ap) or basolateral (Bl) side, as described under MATERIALS AND METHODS. After immunoprecipitation, endogenous proteins gp135 (A) and uvomorulin (B) were detected by ^{125}I-streptavidin blotting. Uvomorulin is predominantly labeled from basolateral side and gp135 from apical side in both control and virus-infected cultures. Identity of >200 kDa protein that coprecipitates with gp135 has not been determined.

After agonist binding, activated TRH receptors stimulate the formation of IP second messengers (6). We measured TRH stimulation of IP formation in MDCK cells after infection with AdCMVmTRHR to demonstrate that the expressed TRH receptors are functionally coupled to second messenger effectors. Subconfluent cultures of MDCK cells (2×10^5 cells/well), prelabeled with *myo*-[^{3}H]inositol, were infected with 10 PFU/cell AdCMVmTRHR, and the dose-response effect of MeTRH was examined (Fig. 2*B*). Increasing concentrations of agonist promoted the formation of increasing amounts of IPs, and half-maximal stimulation was achieved using 1 nM MeTRH. Thus these initial experiments demonstrate that MDCK cells can be infected with human adenovirus constructs and can express TRH receptors that bind ligand and couple to appropriate intracellular effector molecules.

Polarized MDCK cells were infected with replication-defective adenoviruses to determine whether this gene transfer technique compromised the integrity or polarity of the epithelial monolayer. No gross cytopathic effects have been observed in cells infected with multiplicities as high as 1,000 PFU/cell. The functional integrity of tight junctions was evaluated by monitoring the transepithelial electrical resistance of MDCK cells cultured on Transwell filters before, immediately, and 24 h after application of recombinant adenovirus. Control and virus-infected filters had similar transepithelial resistance values of 247 ± 22 and 236 ± 14 $\Omega \cdot cm^2$, respectively. Monolayer integrity was also verified during individual binding experiments by sampling aliquots from apical and basolateral compartments for transepithelial leakage of [*methyl*-^{3}H]TRH. In all cases, this was found to be <2% per hour. Finally, no difference in the polarized distribution of an apical (gp135) or a basolateral (uvomorulin) membrane marker was observed between control and infected cells (Fig. 3).

TRH receptors are primarily expressed on the basolateral surface of polarized MDCK cells and a variety of other epithelial cell types. The steady-state distribution of TRH receptors on polarized MDCK cells was determined by quantifying specific binding of radiolabeled ligand applied to apical or basolateral membrane domains. A time course of [*methyl*-^{3}H]TRH association shows that the ligand binds to sites on both surface domains (Fig. 4). Binding to apical and basolateral membranes follows similar kinetics, and equilibrium is reached after 30 min. Saturation binding experiments were performed to determine the number and binding affinity of these sites (Fig. 5). Scatchard analysis of these data reveals that TRH receptors exhibit a single type of binding site on either membrane domain, with an apparent K_d for MeTRH of 0.85 ± 0.2 and 1.1 ± 0.2 nM for the apical and basolateral sites, respectively. Comparison of B_{max} values shows that TRH receptors are expressed predominantly (>80%) on the basolateral cell surface. Under the infection conditions employed, MDCK cells express 1.8×10^4 apical TRH receptors and 7.8×10^4 basolateral TRH receptors.

To determine whether the distribution of TRH receptors changes with time after the onset of receptor synthesis, filter-grown MDCK cultures were infected with AdCMVmTRHR and TRH receptors expressed at the apical and basolateral surface were quantified by

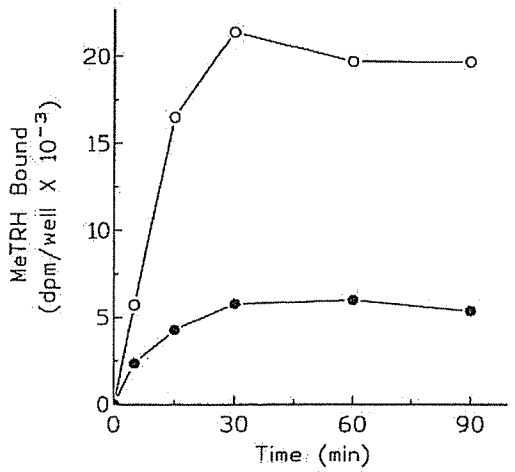


Fig. 4. Time course of [*methyl*-^{3}H]TRH binding to apical (●) and basolateral (○) surfaces of MDCK cells expressing TRH receptors. Polarized MDCK cells on Transwells were infected with 10 PFU/cell AdCMVmTRHR. After 18 h, 1 nM [*methyl*-^{3}H]TRH in HEPES buffer was applied to either apical or basolateral cell surface for indicated times at 37°C and specific binding was measured as described under MATERIALS AND METHODS. Each point represents mean of triplicate determinations of specific binding.

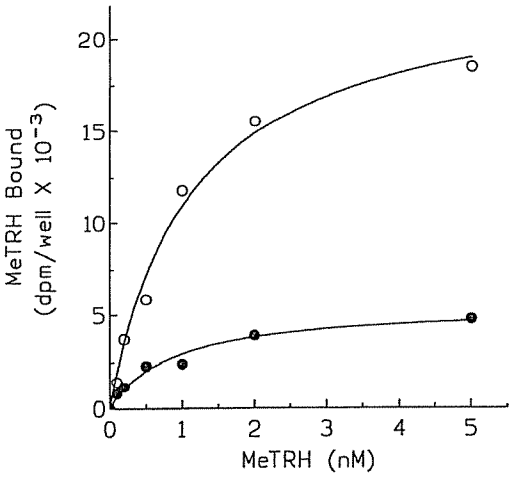


Fig. 5. Equilibrium binding of [*methyl*-^{3}H]TRH to apical (●) and basolateral (○) surfaces of MDCK cells expressing TRH receptors. Polarized MDCK cells on Transwells were infected with 7 PFU/cell AdCMVmTRHR. After 18 h, indicated concentrations of [*methyl*-^{3}H]TRH were applied in presence or absence of 1 μM unlabeled MeTRH to either apical or basolateral cell surface. Incubations were performed at 37°C for 1 h. Each point represents mean of duplicate determinations of specific binding.

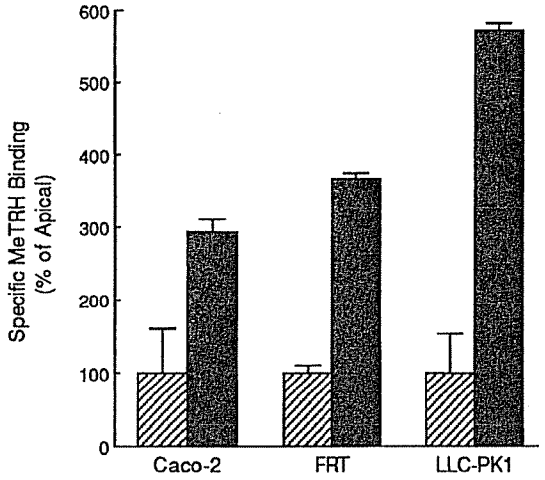


Fig. 7. TRH receptors accumulate on basolateral surface of several types of epithelial cells. Polarized human intestinal epithelial cells (Caco-2), Fischer rat thyroid (FRT), and porcine kidney epithelial (LLC-PK_1) cells on Transwells were infected with 10 PFU/cell AdCMVmTRHR. After 24 h, 1 nM [*methyl*-^{3}H]TRH in HEPES buffer was applied to either apical (hatched bars) or basolateral (solid bars) cell surface for 60 min at 37°C, and specific binding was measured as described under MATERIALS AND METHODS. Bars represent spread between duplicate determinations in a representative experiment.

radiolabeled ligand binding analysis 6, 12, 18, and 24 h later. Expression was evident after 6 h, the earliest time studied, at which time ~85% of the surface-expressed receptors were present on the basolateral domain (Fig. 6). With increasing incubation time, the expression level of the TRH receptor rises gradually and reaches a peak level ~24 h after adenovirus infection. During this time, the majority of TRH receptors continue to accumulate on the basolateral surface, although a smaller but significant fraction of receptors accumulates on the apical cell surface. At 12, 18, and 24 h, the percentage of TRH receptors on the basolateral membrane domain is 81, 83, and 72%, respectively (Fig. 6). In a separate experiment, cells were incubated for 48 h after virus infection, and the percentage of TRH receptors on the basolateral surface was determined to be 81%. The same distribution of TRH receptors is observed when ligand binding analysis is performed at 0°C, indicating that the observed polarity does not reflect a redistribution of receptors after application of ligand at 37°C (Fig. 6). Therefore, after infection of MDCK cells with moderate numbers of AdCMVmTRHR, the TRH receptor is predominantly expressed on the basolateral surface of MDCK cells at all times examined.

The same adenovirus vector has been used to study the distribution of TRH receptors in several other polarized epithelial cell types, including Fischer rat thyroid (FRT), human intestinal epithelial cells (Caco-2), and porcine kidney epithelial cells (LLC-PK_1). In each of these cell types, TRH receptors accumulate predominantly (between 80 and 85%) on the basolateral surface, although a smaller but significant fraction of receptors is detected on the apical surface domain (Fig. 7). Therefore, mechanisms leading to basolateral

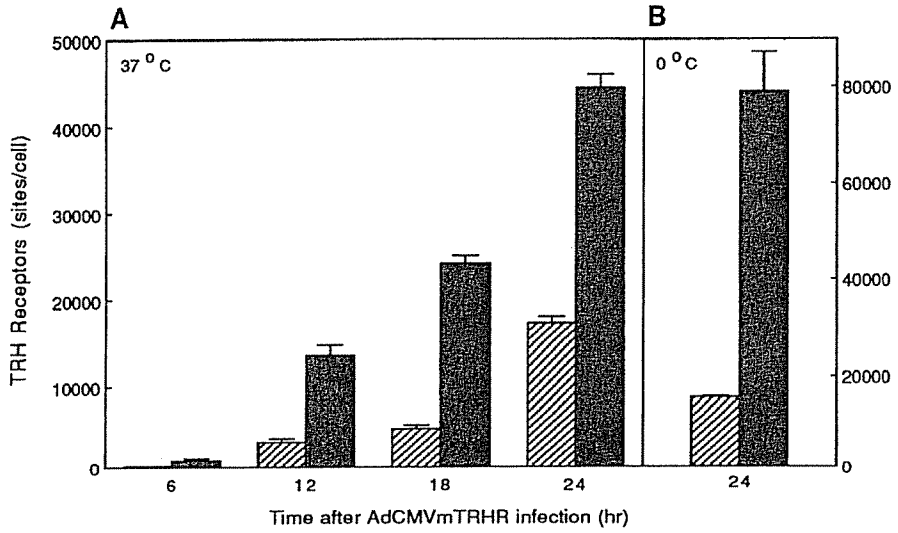


Fig. 6. Distribution of TRH receptors on apical (hatched bars) and basolateral (solid bars) membranes of MDCK cells with increasing duration of expression. Polarized MDCK cells on Transwells were infected with 10 PFU/cell AdCMVmTRHR. *A*: cultures were incubated for 6, 12, 18, and 24 h, then 1 nM [*methyl*-^{3}H]TRH in HEPES buffer was applied to either apical or basolateral culture compartment. Incubation was for 1 h at 37°C, and numbers of TRH receptors expressed at apical and basolateral cell surfaces were measured as described under MATERIALS AND METHODS. *B*: cultures were incubated for 24 h, then 1 nM [*methyl*-^{3}H]TRH in HEPES buffer was applied to either apical or basolateral culture compartment. Incubation was for 4 h at 0°C, and specific binding was measured as described under MATERIALS AND METHODS. Bars represent means ± SD of triplicate determinations in a representative experiment.

polarity of TRH receptor expression in MDCK cells appear to be common to other epithelia derived from different tissues and species.

Signals responsible for basolateral polarity and rapid internalization of TRH receptors can be segregated. Basolateral sorting of all type I membrane proteins studied to date is mediated by distinct cytoplasmic determinants (reviewed in Ref. 19). Deletion or mutation of these signals frequently leads to the missorting of cargo proteins to the apical plasma membrane. A major class of sorting determinants is related to the clathrin-coated pit signals that mediate rapid receptor internalization from the plasma membrane. However, it is unclear whether proteins that span the membrane multiple times also utilize endocytic motifs to establish basolateral polarity.

TRH bound to its receptor undergoes a rapid transformation to an acid-resistant form that represents an internalized receptor-ligand complex (1, 10). As an initial effort to determine whether the same signals responsible for rapid internalization of TRH receptors establish the basolateral polarity of these molecules, the distribution of a truncated form of the TRH receptor was assessed (Fig. 1). Previous studies in nonepithelial cell lines have demonstrated that this mutant, C335Stop, which is missing the last 59 amino acids, is poorly internalized following agonist binding (21).

Polarized MDCK cells infected with equal multiplicities of adenovirus vectors encoding wild-type and C335Stop TRH receptors express similar numbers of receptors at the cell surface. In the experiment shown in Fig. 8, cells expressed ~5.4×10^4 native and 3.8×10^4 truncated receptors, indicating that the efficiency of cell surface transport of the truncated receptor is not impaired. Full-length TRH receptors expressed in MDCK cells are rapidly internalized following ligand binding (Fig. 8*A*). Receptors expressed on both the apical and the basolateral surface are internalized with similar kinetics, resulting in roughly 70–80% internalization of MeTRH specifically bound to these cells within 15 min of incubation. In marked contrast, only ~10% of MeTRH specifically bound to C335Stop TRH receptors is internalized from either apical or basolateral membrane domains during 1 h of incubation (Fig. 8*A*). The truncated receptors bind TRH with equal affinity and activate IP second messenger formation from the apical and basolateral surfaces to a similar extent as wild-type TRH receptors (Ref. 21 and Yeaman, Heinflink, Rodriguez-Boulan, and Gershengorn, unpublished data).

The distribution of truncated TRH receptors is indistinguishable from that of wild-type TRH receptors (Fig. 8*B*). After infection of polarized MDCK cells with a replication-defective adenovirus vector encoding the C335Stop TRH receptor, ~83% of the receptors are expressed on the basolateral surface (compared with 81% for wild-type receptors in the experiment shown). Therefore, although signals present in the carboxy-terminal cytoplasmic domain of the TRH receptor are critical for ligand-stimulated internalization of these receptors, the basolateral polarity of these proteins can be established by signals residing elsewhere in the molecule.

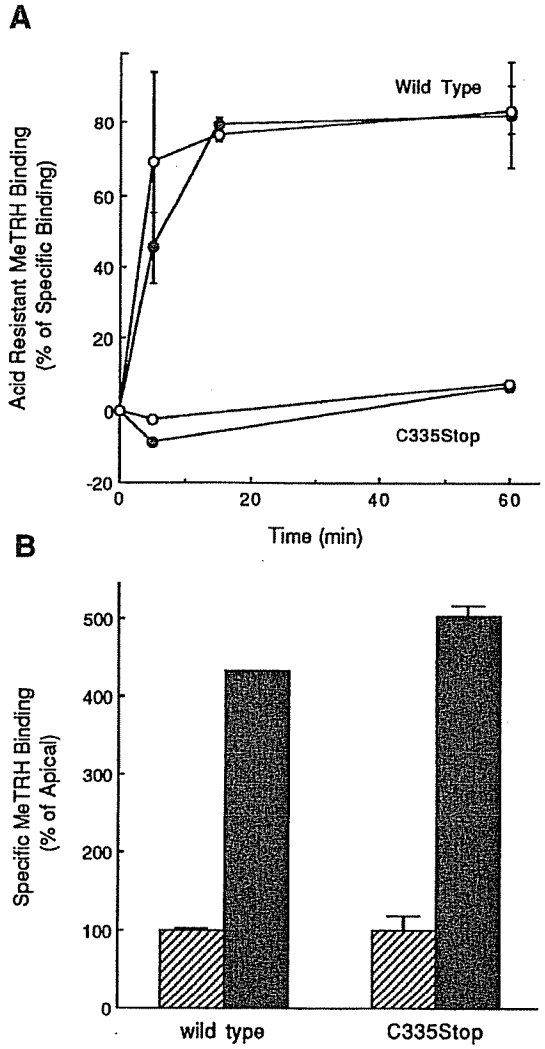


Fig. 8. TRH receptors accumulate on basolateral surface of MDCK cells independently of carboxy-terminal endocytic signals. Polarized MDCK cells on Transwells were infected with 10 PFU/cell AdCMVmTRHR or AdCMV-C335Stop, encoding a TRH receptor (C335Stop) missing last 59 amino acids. *A*: 24 h after infection, cells were incubated at 37°C for times indicated with 1 nM [*methyl*-^{3}H]TRH applied to apical (●) or basolateral (○) surface. Total specific and acid-resistant binding were measured as described under MATERIALS AND METHODS. *B*: cells were incubated at 37°C for 60 min, and total specific binding was measured as above. Hatched bars, apical surface; solid bars, basolateral surface. Bars and data points represent means ± SD of multiple determinations ($n = 6$).

Basolateral polarity of TRH receptor expression is independent of G protein coupling. In addition to the carboxy-terminal tail, TRH receptors possess one other major cytoplasmic domain that may contain basolateral sorting information. The putative third intracellular loop formed between the fifth and sixth transmembrane segments is essential for the coupling of activated receptors to downstream signaling molecules (22). A growing body of evidence suggests that post-Golgi membrane trafficking is regulated by heterotrimeric G proteins (reviewed in Ref. 3). Both $G\alpha_{i\text{-}3}$ and $G\alpha_s$ are implicated in membrane transport, although other

types of heterotrimeric G proteins are also associated with Golgi membranes in certain cell types (15, 25, 28). In rat pituitary epithelial cells, $G\alpha_q$ is found on both the plasma membrane and the Golgi membrane (30). Because activated TRH receptors couple to this G protein, the possible significance of this interaction in the molecular trafficking of the receptor was investigated.

To determine whether basolateral sorting of TRH receptors requires sequences in the third intracellular loop, mutant Δ218–263 TRH receptors were expressed in MDCK cells (Fig. 1). Because an adenovirus vector encoding this mutant has not been produced, stable MDCK clones expressing the Δ218–263 TRH receptor were generated. MDCK cell lines expressing wild-type TRH receptors were also generated to permit comparison with clones expressing the mutant receptor and with results obtained using the adenovirus vector encoding the native receptor. Mutant Δ218–263 TRH receptors lack essentially all of the third intracellular loop, and, although these receptors are efficiently transported to the cell surface and bind TRH specifically ($\sim 1.9 \times 10^5$ sites/cell), they do not produce IPs when exposed to a supramaximal (1 μM) concentration of TRH (Fig. 9*A*). This is due to the receptor mutation and not to a defect elsewhere in the signal transduction pathway in this clone. Thus introduction by adenovirus-mediated gene transfer of wild-type TRH receptors into cells expressing Δ218–263 TRH receptors restores the response (Fig. 9*A*).

Although the primary signals for agonist-induced internalization are present in the TRH receptor carboxy-terminal tail, efficient endocytosis requires the presence of the third intracellular loop, presumably because internalization requires coupling to G proteins and phospholipase C (22). The extent of receptor internalization following MeTRH binding to mutant Δ218–263 TRH receptors is significantly reduced compared with native TRH receptors. Whereas MDCK clones expressing wild-type receptors internalize roughly 70–80% of specifically bound MeTRH during a 1-h incubation, cells expressing Δ218–263 TRH receptors internalize only 30–40% of specifically bound MeTRH (Fig. 9*B*).

Considering the dramatic effects that deletion of the third intracellular loop has on the function and endocytosis of TRH receptors, it is somewhat remarkable that this mutation does not significantly alter the distribution of TRH receptors at the cell surface. The Δ218–263 TRH receptors are predominantly (>92%) localized on the basolateral plasma membrane of stable MDCK clones (Fig. 9*C*). This distribution is indistinguishable from that observed for wild-type TRH receptors, 94% of which accumulate on the basolateral surface (Fig. 9*C*). Therefore, although removal of the third intracellular loop effectively "kills" the TRH receptor by uncoupling it from downstream signaling molecules, the accumulation of receptors on the basolateral surface can occur in the absence of this domain.

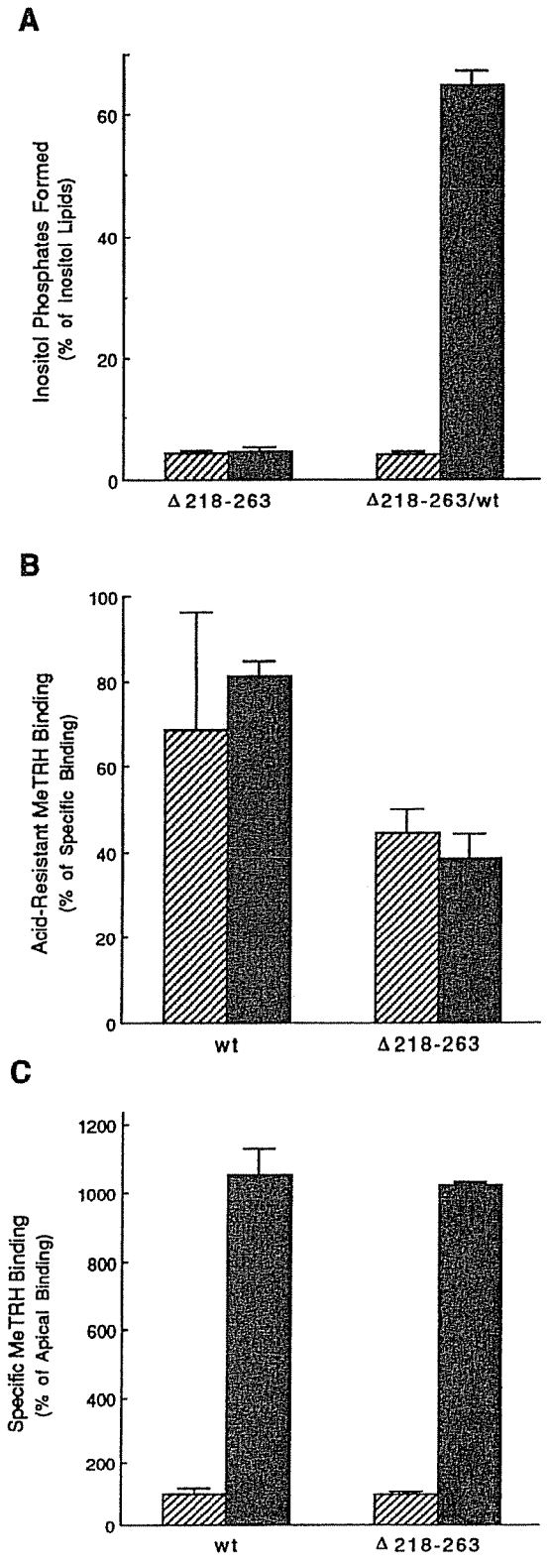
DISCUSSION

The main objectives of this study were to determine whether TRH receptors are expressed in a polarized fashion by MDCK cells following adenovirus-mediated gene transfer and to begin dissecting the molecular basis for the trafficking of G protein-coupled receptors in these cells. The distribution of TRH receptors was determined in binding assays using the high-affinity TRH analogue [*methyl*-^{3}H]TRH. In polarized MDCK cells, TRH receptors are predominantly expressed on the basolateral surface. This distribution is observed from the onset of TRH receptor expression and is maintained for at least 2 days after adenovirus-mediated gene transfer. Moreover, basolateral polarity of TRH receptor expression is unchanged on deletion of the receptor's cytoplasmic carboxy terminus that contains its major endocytic motif. This is the first demonstration that endocytic and basolateral sorting signals can be segregated in a G protein-coupled receptor. Finally, removal of the large third intracellular loop does not alter the polarized distribution of TRH receptors. Thus basolateral delivery of TRH receptors occurs independently of sequences required for coupling activated receptors to downstream signaling molecules. These results suggest that molecular sorting of seven transmembrane-spanning G protein-coupled receptors may require signals that are more complex than a linear sequence of amino acids in the carboxy-terminal cytoplasmic tail, such as those identified in many type I membrane proteins. It is even possible that sorting information resides in other domains of these receptors, such as the membrane-spanning segments or the extracellular domain. Clearly, it is important to study the molecular trafficking of many members of this family to gain insight into this complex sorting event. Development of recombinant adenovirus vectors to gain expression of these molecules in multiple epithelial cell types represents a major step toward this goal.

Although basolateral sorting signals in seven transmembrane-spanning proteins remain unidentified, we were attracted to signals that mediate rapid receptor endocytosis based on earlier studies of type I membrane proteins. In many such proteins, similar overlapping sequences mediate endocytosis and basolateral targeting (reviewed in Ref. 19). These signals commonly show a strong dependence on either a tyrosine residue or a dileucine motif located upstream of acidic amino acids. Internalization of TRH receptors absolutely requires sequence elements within the carboxy-terminal cytoplasmic domain of the protein. Studies in COS-1 cells have demonstrated that a membrane-proximal cysteine residue (Cys-335 or Cys-337) as well as a domain between residues 360 and 367 are essential for agonist-stimulated internalization of TRH receptors (21). Neither of these motifs resembles previously identified basolateral sorting signals, and their removal does not alter basolateral expression of the receptor. Keefer et al. (12) recently reported that a similar carboxy-terminal truncation failed to disrupt basolateral targeting of the α_{2A}-adrenergic receptor. However, no effects on receptor endocytosis were reported in that study.

Because all but six amino acids of the TRH receptor cytoplasmic domain have been deleted in the C335Stop

mutant, it is likely that basolateral sorting information resides elsewhere in this protein. The third intracellular loop is a large cytoplasmic domain that is indispensable for TRH receptor function. Deletion of this domain uncouples the receptor from downstream effectors and reduces the efficiency of agonist-stimulated internalization. This domain is also notable due to the presence of an isoleucine-leucine motif potentially involved in basolateral sorting. However, deletion of all but four amino acids of this loop does not affect the basolateral accumulation of TRH receptors. Therefore, basolateral sorting of TRH receptors requires neither the functional coupling of receptors with G proteins nor structural information residing in the endofacial amino acid stretch within the third intracellular loop.



Two possible explanations may account for basolateral accumulation of TRH receptors in the absence of the two major cytoplasmic domains. First, sorting information may reside in the transmembrane or extracellular domains. One possible candidate is the sequence NPXXY that is buried within the putative seventh transmembrane domain. A closely related sequence, NPXY, when present in the cytoplasmic domain of the low-density lipoprotein receptor, is essential for both endocytosis and basolateral sorting (19). However, no role for this sequence has been defined in the TRH receptor, and it is unclear how potential sorting information would be decoded from within the lipid bilayer. On the other hand, this sequence motif is highly conserved by many members of the G protein-coupled receptor family, and substitution of alanine for tyrosine in the β_2-adrenergic receptor abolishes agonist-stimulated internalization (2). However, the same alanine substitution in the α_{2A}-adrenergic receptor fails to disrupt basolateral targeting (12).

Second, there may be redundancy in sorting information encoded by the TRH receptor. It is possible that the carboxy-terminal tail and the third intracellular loop possess independently acting sorting signals. In this case, removal of one or the other alone would not affect the polarity of receptor expression, but removal in combination would disrupt sorting. It is important to note that truncation of cytoplasmic sequences in the carboxy-terminal tail and large third intracellular loop individually or in combination does not alter the sorting of the α_{2A}-adrenergic receptor (12). Together with the findings presented here, these results imply that transmembrane sequences are critical for sorting of multispanning receptors. However, it is unlikely that the overall topology assumed by a protein that spans the membrane multiple times directs these molecules to the basolateral membrane, since several polytypic membrane proteins are restricted to the apical domain

Fig. 9. Basolateral polarity of TRH receptor expression is unchanged on deletion of third intracellular loop. *A*: stably transfected MDCK II cells expressing Δ218–263 TRH receptors were infected or mock infected with 10 PFU/ml AdCMVmTRHR. After prelabeling with 1 μCi/ml *myo*-[^{3}H]inositol for 24 h, MeTRH stimulation of IP formation in cells expressing only mutant receptors (Δ218–263) or coexpressing mutant and wild-type receptors (Δ218–263/wt) was measured as described under MATERIALS AND METHODS. Hatched bars, basal; solid bars, +TRH. *B* and *C*: stable MDCK II clones expressing either wild-type (wt) or mutant Δ218–263 TRH receptors were cultured on Transwells for 4–5 days. Total specific and acid-resistant binding were quantified after application of 1 nM [*methyl*-^{3}H]TRH to either apical (hatched bars) or basolateral (solid bars) cell surface for 60 min at 37°C. Bars represent means ± SD of triplicate determinations in a representative experiment.

(8, 18, 24). Sorting of these molecules more likely involves recognition of specific amino acid sequences within the transmembrane segments or of sorting motifs that were not affected by prior mutagenesis efforts.

In conclusion, adenovirus-mediated gene transfer appears to be an excellent method for the controlled expression of TRH receptors in polarized MDCK cells. A number of advantages over transfection protocols are clear. First, the expression level of the receptor is directly proportional to the multiplicity of infection, thus permitting easy manipulation of gene expression. Also, use of adequate numbers of viruses ensures that most or all the cells in the population express the gene of interest. In principle, it should be possible to coinfect cells with multiple distinct adenovirus vectors to achieve coordinated expression of two or more proteins in the same cells. This approach could be extended for expression of other markers of the apical and basolateral secretory pathway in MDCK cells and other polarized cell systems. Most importantly, because recombinant adenovirus vectors are replication defective, cytopathic effects encountered with live viruses, such as influenza and vesicular stomatitis virus, are avoided. To date, we have observed no deleterious effects of adenovirus vectors on the epithelium. Thus infection of cells with as many as 1,000 PFU/cell does not appear to compromise the integrity of tight junctions or alter the polarized distribution of apical and basolateral membrane marker proteins. Continued study of the optimum conditions for adenovirus infection of polarized epithelial cells in culture and of the long-term effects on cell structure and function will also have important implications for the use of these vectors in human gene therapy.

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Modulation of human erg K^+ channel gating by activation of a G protein-coupled receptor and protein kinase C

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1. Modulation of the human *ether-à-go-go*-related gene (HERG) K^+ channel was studied in two-electrode voltage-clamped *Xenopus* oocytes co-expressing the channel protein and the thyrotropin-releasing hormone (TRH) receptor.

2. Addition of TRH caused clear modifications of HERG channel gating kinetics. These variations consisted of an acceleration of deactivation, as shown by a faster decay of hyperpolarization-induced tail currents, and a slower time course of activation, measured using an envelope of tails protocol. The voltage dependence for activation was also shifted by nearly 20 mV in the depolarizing direction. Neither the inactivation nor the inactivation recovery rates were altered by TRH.

3. The alterations in activation gating parameters induced by TRH were demonstrated in a direct way by looking at the increased outward K^+ currents elicited in extracellular solutions in which K^+ was replaced by Cs^+.

4. The effects of TRH were mimicked by direct pharmacological activation of protein kinase C (PKC) with β-phorbol 12-myristate,13-acetate (PMA). The TRH-induced effects were antagonized by GF109203X, a highly specific inhibitor of PKC that also abolished the PMA-dependent regulation of the channels.

5. It is concluded that a PKC-dependent pathway links G protein-coupled receptors that activate phospholipase C to modulation of HERG channel gating. This provides a mechanism for the physiological regulation of cardiac function by phospholipase C-activating receptors, and for modulation of adenohypophysial neurosecretion in response to TRH.

The human *ether-à-go-go* (*eag*)-related gene (HERG) encodes a K^+ channel (the HERG channel) that constitutes the molecular basis of the cardiac repolarizing K^+ current, I_{Kr} (Sanguinetti *et al.* 1995; Trudeau *et al.* 1995). Malfunction of HERG channels is the cause of both inherited and acquired long-QT syndromes, characterized by an unusually slow repolarization of cardiac action potentials leading to cardiac arrhythmia and eventually ventricular fibrillation and sudden cardiac death (Curran *et al.* 1995; Sanguinetti *et al.* 1995; Spector *et al.* 1996*a*). Furthermore, HERG channels are molecular targets for widely used pharmacological agents such as class III anti-arrhythmics (Spector *et al.* 1996*a*) and some histamine receptor antagonists (Suessbrich *et al.* 1996). HERG channels were initially isolated from hippocampus, but their role in neuronal function is not completely understood. However, they have been implicated in the changes of the resting membrane potential associated with the cell cycle and in the control of neuritogenesis and differentiation in neuronal cells (Arcangeli *et al.* 1993, 1995; Faravelli *et al.* 1996). Finally, a recent report by Chiesa *et al.* (1997) indicated an important role for HERG channels in neuronal spike-frequency adaptation.

In spite of the physiological importance of HERG channels, little is known about their regulation by different neurotransmitters and/or hormone receptors. In GH_3 rat anterior pituitary cells, regulation of an inwardly rectifying K^+ current constitutes an important point for control of pacemaker activity in response to thyrotropin-releasing hormone (TRH; Barros *et al.* 1994, 1997). Such a regulation is exerted by means of a phosphorylation/dephosphorylation cycle triggered by a still unknown protein kinase, which is specifically reverted by protein phosphatase 2A (Barros *et al.* 1992, 1993; Delgado *et al.* 1992). Recent kinetic and pharmacological evidence indicates that a HERG-like K^+ channel is the cause of the TRH-regulated inwardly rectifying K^+ currents (Barros *et al.* 1997). The availability of cloned TRH receptors (TRH-Rs) and HERG channels

allowed us to develop an *in vitro* assay to study the mechanism(s) of HERG regulation by co-expression of receptor and channel proteins.

Expression of HERG product in *Xenopus* oocytes yields depolarization-activated K^+ currents which, as for GH_3 cell currents, show strong inward rectification (Sanguinetti *et al.* 1995; Trudeau *et al.* 1995; Schönherr & Heinemann, 1996; Spector *et al.* 1996*b*; Wang *et al.* 1996, 1997). Recently it has been shown that this rectification arises from a C-type rapid inactivation mechanism (Schönherr & Heinemann, 1996; Smith *et al.* 1996; but see Wang *et al.* 1996, 1997) that reduces conductance at positive voltages and strongly limits the level of outward current after depolarizing the membrane. This precludes an accurate estimation of activation and inactivation parameters from direct measurements of outward currents, in which activation and inactivation properties overlap. In this report, we performed a characterization of the HERG gating properties by using an envelope of tail currents protocol. Both in oocytes and adenohypophysial cells, activation of phospholipase C (PLC) and generation of the two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) are the prototypical consequences of TRH-R activation (de la Peña *et al.* 1992; Corette *et al.* 1995; Gershengorn & Osman, 1996). Our results with oocytes co-expressing HERG and TRH-R demonstrate clear alterations of HERG channel gating by TRH. Such alterations are manifested as an acceleration of deactivation and a slower time course of channel activation without any significant change in inactivation or inactivation recovery rates. The parallel between the effects of TRH and the protein kinase C (PKC)-specific activator β-phorbol 12-myristate,13-acetate (PMA) indicates that a PKC-dependent pathway links the TRH-R to modulation of HERG. Our data also indicate that a phosphorylation triggered by activation of PKC is able to regulate channel gating properties by G protein-coupled receptors that generate PLC-dependent signals.

METHODS

Microinjection and electrophysiology of oocytes

Mature female *Xenopus laevis* (Nasco, Fort Atkinson, WI, USA) were anaesthetized by immersion in benzocaine solutions and subsequently maintained on ice in order to obtain oocytes. Ovarian lobes were removed through a small incision in the abdominal wall. After removal of the ovarian lobe, the frogs were sutured in the abdominal wall and in the external skin, and allowed to recover in a small water-filled container, with their heads elevated above water level. Once the animal had recovered from anaesthesia, it was placed in a separate aquarium by itself and periodically monitored until healed. Typically, lobes were obtained two or three times from a single frog, with several months in between. When individual frogs no longer yielded acceptable oocytes, anaesthetized frogs were killed by an overdose of benzocaine.

Procedures for microinjection and two-electrode voltage-clamp of oocytes have been described elsewhere (de la Peña *et al.* 1992, 1995; del Camino *et al.* 1997). Oocytes were maintained in OR-2 medium (mM: 82·5 NaCl, 2 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1 Na_2HPO_4 and 10 Hepes, at pH 7·5). Cytoplasmic microinjections were performed with 30–50 nl of *in vitro* synthesized cRNA per oocyte. HERG currents and TRH-induced responses were studied in manually defolliculated oocytes (de la Peña *et al.* 1992) using extracellular high-K^+ OR-2 medium in which 50 mM KCl was substituted for an equivalent amount of NaCl. When indicated, a Cs^+-containing extracellular solution was used constituted from OR-2 medium without K^+, and in which NaCl was substituted with an equivalent amount of CsCl.

Functional expression was typically assessed 2–3 days after microinjection. Current measurements were performed at room temperature (20–25 °C) using the two-electrode voltage-clamp method with a Turbo TEC 01C (NPI, Tamm, Germany) amplifier and 2 M KCl-filled glass capillaries showing DC resistances between 0·5 and 2·0 MΩ. Current recordings were obtained in an experimental chamber of 0·2 ml volume continuously perfused at 4–5 ml min^{-1}. Membrane potential was clamped at −80 mV.

Channel deactivation and inactivation recovery kinetics were obtained from tail currents upon membrane repolarization, following depolarizing pulses that displaced the channels to open and inactive states. Repolarizations following depolarizing pulses lasted for 1–5 s to ensure total relaxation of tail currents and proper fits, but only the initial portion of the tails is shown in some graphs for clarity. By fitting an exponential to the initial rising phase and a second one to the decaying portion of the tails, both the rate of recovery from inactivation and the deactivation rate were measured, respectively.

The simultaneous occurrence of closed to open and open to inactive transitions at positive voltages due to the fast rate of HERG channel inactivation precludes an accurate estimation of activation and inactivation kinetic parameters by looking at the time-dependent development of outward currents. As an alternative, the time course of transitions from closed to open plus inactive states upon depolarization was monitored indirectly with an envelope of tail currents protocol, varying the duration of the depolarization pulse, fitting the relaxation of the tail currents recorded once the membrane is stepped back to a negative voltage, and extrapolating the current magnitude to the moment the depolarizing pulse was ended (Trudeau *et al.* 1995).

The time dependence of HERG fast inactivation from the open state can be determined by a dual-pulse protocol (Smith *et al.* 1996; Spector *et al.* 1996*b*; Schönherr & Heinemann, 1996) in which after a depolarization pulse to activate (and inactivate) the channels, the membrane is hyperpolarized briefly to allow them to recover from inactivation, entering the open state. Once the maximum inward current is obtained at negative voltages, the membrane is again depolarized to re-inactivate the channels. This re-inactivation is seen as a single exponential decline in current along the second depolarization pulse, from which the rate of the open to inactive transition can be obtained.

The data acquisition and analysis were performed using Apple Macintosh computers with Pulse/PulseFit (HEKA, Lambrecht, Germany) and IGOR (Wavemetrics, Lake Oswego, OR, USA) software. The output of the amplifier was digitized at 1 kHz. Records are shown without leak and capacitive correction unless otherwise indicated. In some cases, a $P/4$ protocol was performed for leak and capacitive current subtraction with a leak holding potential of −80 mV and a scaling factor of −0·2. Because the efficiency of the channel and receptor protein expression varied among oocyte batches, the data collected for the source batch of oocytes were compared for each set of experiment in the graphs. Nevertheless, the experiments were repeated at least three times

with different batches from different donors. Data are presented as means ± S.E.M.

Plasmids and preparation of cRNA

Isolation of the TRH-R cDNA has been described previously (de la Peña *et al.* 1992). The plasmid containing the cDNA for the HERG channel was generously provided by Dr E. Wanke (University of Milano, Italy). Plasmids were linearized and capped cRNA was synthesized *in vitro* from the linear cDNA templates by standard methods using T7 or SP6 RNA polymerases as described (de la Peña *et al.* 1992).

Chemicals

TRH, PMA, KN-62, benzocaine and genistein were purchased from Sigma. GF109203X was from Calbiochem (San Diego, CA, USA). All other reagents were purchased from Sigma and were the highest quality available.

RESULTS

Effect of TRH-R activation on HERG channel gating transitions studied in K^+-containing solutions

To determine whether the HERG channels may be modulated by receptor pathways that regulate PLC-dependent signals, we co-expressed the HERG channel protein with the TRH-R from rat adenohypophysial cells (de la Peña *et al.* 1992). Treatment of oocytes with 1 μM TRH resulted in fast activation of transient inward Cl^- currents when the oocyte membrane was maintained at −80 mV (Fig. 1*A*, left). These currents, due to IP_3-triggered release of Ca^{2+} from intracellular stores, were indistinguishable from those measured in oocytes without HERG channel transcripts (Fig. 1*B*, left; see de la Peña *et al.* 1992, 1995; del Camino *et al.* 1997). To test for receptor-mediated modulation of HERG currents, the co-injected oocytes clamped at −80 mV were submitted to a 400 ms depolarization at regular intervals followed by a repolarization to −100 mV. Without TRH addition, HERG-expressing oocytes bathed in solutions containing 50 mM K^+ displayed only small outward currents (Fig. 1*A*, right). On repolarization to a negative membrane potential, a slow inward tail current is obtained with an initial rising phase as a result of fast inactivation removal, followed by a slow decay due to channel closing.

Application of TRH caused a transient enhancement of outward currents recorded upon depolarization and of hyperpolarization-evoked inward tail currents. The transiently enhanced currents correspond to the Ca^{2+}-activated Cl^- currents triggered by the sudden release of Ca^{2+} from intracellular stores (see above). However, 1 or 2 min after the introduction of TRH, the hyperpolarization-evoked tail currents were obviously reduced (Fig. 1*A*, right). Such a reduction was mainly shown by a marked acceleration of decay. It is important to note that the reduction in tail currents was detected even before the basal current at −80 mV completely returned to its initial magnitude. However, an increased Cl^- permeability in response to TRH would yield a bigger current at the negative voltages at which the tail currents are recorded. Further evidence that TRH-induced increases of oocyte Cl^- conductance do not contribute significantly to the observed effects on the tail currents are as follows. (i) The modifications of the inward tail current kinetics lasted long after the initial Ca^{2+}-dependent Cl^- current increase was almost completely reverted (trace *b* in Fig. 1*A*). (ii) Delayed development of IP_3-induced Cl^- currents is associated with appearance of slowly rising non-inactivating hyperpolarization-evoked inward currents (Hartzell, 1996). This would cause an increase in the size of the tail currents and a slower rate of decay. However, the opposite effect is seen, and (iii) in oocytes expressing only TRH-Rs, 2 or 3 min after TRH addition the membrane currents recorded at −100 mV consisted exclusively of an instantaneous small inward current followed by a fast mono-exponential decay (Fig. 1*B*). This yields almost undetectable currents over the time range at which deactivation of HERG tail currents takes place. Altogether, this indicates that although the modifications induced by TRH treatment can be even larger than observed, the alterations in tail current kinetics are due to variations in the deactivation gating of the expressed HERG channels.

The results shown in Fig. 1*A* and *C* indicate that in spite of a certain variability in the kinetic parameters obtained from different oocytes, addition of TRH reduced the deactivation time constant by nearly a half (see also Table 1). The acceleration of the current deactivation was reversed several minutes after washing out TRH from the recording chamber. Interestingly, the reversion of the TRH effect was usually less than 50% during an exposure to the neuropeptide lasting up to 10 min. Furthermore, although a short (20–30 s) exposure to the hormone was systematically followed by a quite rapid recovery of the initial rates of tail current decay, only a slow and partial recovery was sometimes achieved following addition of TRH for several minutes (not shown).

HERG channel deactivation is a voltage-dependent process that occurs at a faster rate when the membrane potential is set at more negative voltages (Sanguinetti *et al.* 1995; Schönherr & Heinemann, 1996; Spector *et al.* 1996*b*). We measured a deactivation time constant of nearly 1 s at −60 mV, which was reduced to 20 ms at −140 mV. This time constant was 50% smaller in the presence of TRH at all voltages between −60 and −140 mV (data not shown).

As shown in Table 1, the rate of HERG recovery from inactivation was not significantly changed by TRH. Thus only minor variations in the time constant of the tail current rising phase were obtained in the presence of the hormone. A similar lack of effect was obtained at all voltages between −60 and −140 mV, at which the recovery process was studied (data not shown).

Due to superposition of relatively slow activation transitions and particularly fast inactivation rates upon depolarization, rigorous estimations of HERG activation rates can only be indirectly achieved by using an envelope of tail currents protocol (see Methods). As shown in Fig. 2, we obtained clear sigmoidal activation kinetics using this procedure.

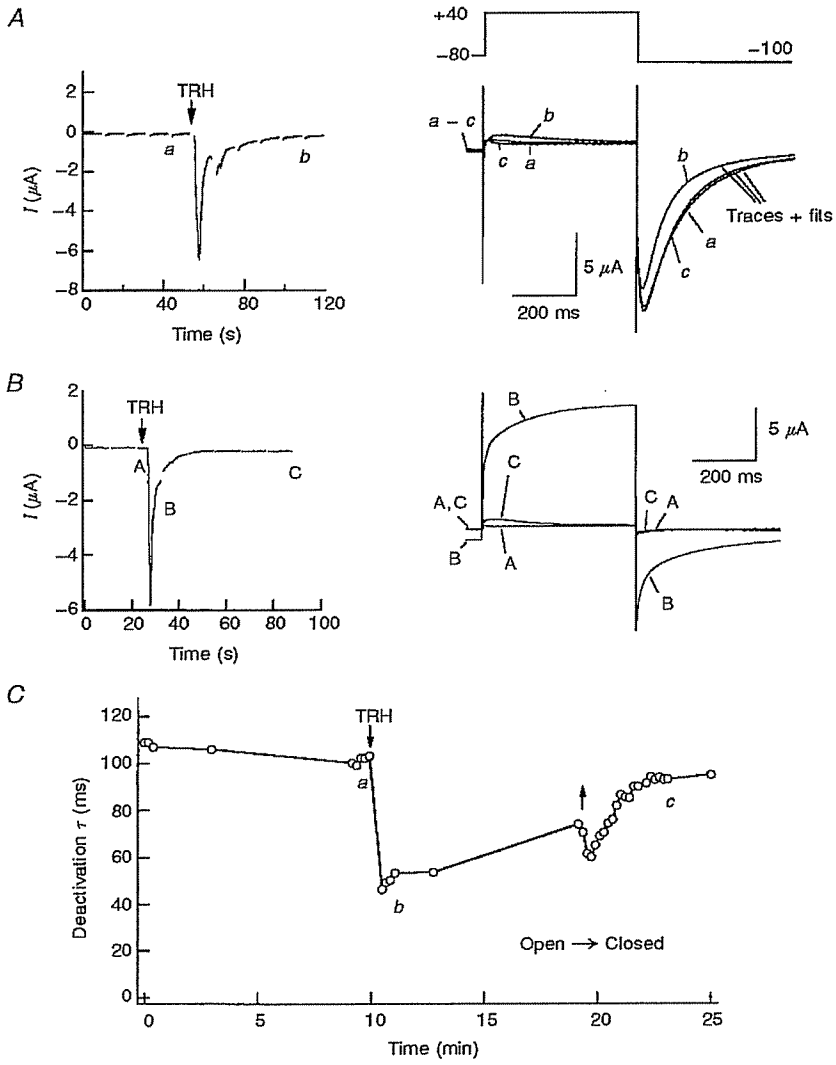

Figure 1. Effect of TRH-R activation on HERG tail current kinetics

A, left, inward Ca^{2+}-dependent Cl^- currents in response to TRH. Representative inward Cl^- currents in a voltage-clamped oocyte 2 days after co-injection of TRH-R and HERG channel cRNA are shown. The start of perfusion with high-K^+ OR-2 plus 1 μM TRH is indicated. The current trace represents continuous segments obtained at −80 mV. The gaps on the trace indicate times at which a depolarization pulse was applied according to the voltage pulse diagram shown on the right. Right, membrane currents elicited by membrane depolarization at different times before (trace *a*) and after challenging the cell with TRH (traces *b* and *c*). Depolarization steps of 400 ms from −80 to +40 mV were delivered to the oocyte every 10 s, followed by a 400 ms repolarization to −100 mV. Membrane currents shown correspond to pulses delivered either 10 s before TRH addition (trace *a*), or 50 s after starting perfusion with 1 μM TRH (trace *b*), respectively. A current trace elicited with an identical protocol after 6 min of TRH washout (trace *c*) is also shown to illustrate the reversibility of the TRH effect. The TRH application lasted for about 9 min. Bi-exponential fits superimposed to the current traces are shown for −100 mV tails. Note the correspondence of current kinetics along the tail rising phase for control and TRH traces. *B*, left, inward Ca^{2+}-dependent Cl^- currents obtained in an oocyte injected with TRH-R cRNA but not with HERG channel messages. Right, membrane currents elicited by membrane depolarizations at the times marked A to C on the left. Identical conditions to those indicated in *A* were used. Note the fast monoexponential decay of the Cl^- tail currents and their small magnitude 1 min after start of the TRH addition. *C*, time course of deactivation time constant variations in response to TRH. The time constant was quantified by fitting a double exponential to the tail as shown in *A* (right). Subsequently, the plotted deactivation time constant and the time constant for inactivation recovery were obtained from the decaying portion and the initial rising phase of the tail, respectively. The periods without data points correspond to times at which *I–V* curves were generated. Time constant values for the two pulses following introduction of TRH in the chamber, corresponding to periods of huge increases in inward Cl^- currents, have not been included in the graph. Start of perfusion with 1 μM TRH and hormone washout is signalled by arrows. *a–c* correspond to the experimental times at which current traces marked with the same lettering in *A* were obtained.

Table 1. Effect of thyrotropin-releasing hormone on HERG kinetic parameters

Treatment	External solution	Activation $t_{1/2}$ (ms)	n	N	Deactivation τ (ms)	Inactivation recovery τ (ms)	n	N
Control	50 mM K^+	30·6 ± 3·5	21	3	589 ± 64	9·4 ± 0·4	48	7
TRH	50 mM K^+	66·0 ± 10·0*	21	3	348 ± 30†	10·4 ± 0·5	48	7
Control	Cs^+	13·3 ± 0·8	3	—	100 ± 4	14·2 ± 2·6	3	—
TRH	Cs^+	19·5 ± 1·8*	3	—	64 ± 3*	12·6 ± 2·7	3	—

* $P < 0.05$; † $P < 0.001$ *vs.* control; Student's paired t test. Times for half-maximal current activation ($t_{1/2}$) were obtained from envelope of tail currents following the voltage protocol shown at the top of Fig. 2. Deactivation and inactivation recovery time constants (τ) were measured from bi-exponential fits to tail currents after repolarizing the membrane to −100 mV. n represents the number of oocytes used for the number of donors indicated by N.

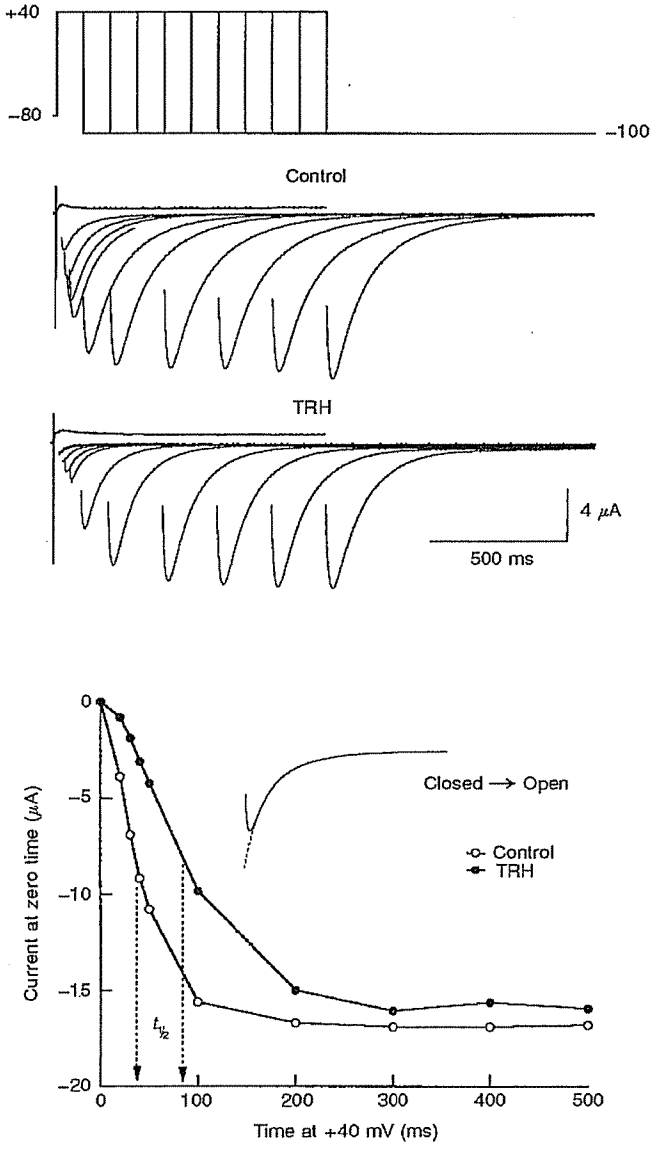

Figure 2. Effect of TRH on HERG channel activation kinetics

The time course of voltage-dependent activation was studied in the absence (control) or the presence of 1 μM TRH by varying the duration of a depolarizing prepulse to +40 mV according to the voltage protocol shown at the top. Test pulses were applied once every 20 s. For TRH, data collection started 2 min after challenging the cell with the neuropeptide. The magnitude of the instantaneous tail current at −100 mV was determined by fitting an exponential to the decaying portion of the tail as shown in the inset of the lower panel, and extrapolating the current to the moment the depolarizing pulse was ended. Capacitive transients have been blanked for clarity. Note the sigmoidal activation kinetics and the shift in the time necessary to attain a half-maximal current magnitude. For further explanation, see text.

Furthermore, the time necessary to attain a half-maximal current magnitude was increased about twofold after treatment of the oocytes with TRH (Fig. 2 and Table 1).

The voltage dependence of the gating transitions during the depolarization pulses can be studied following a similar protocol in which the membrane potential is stepped to different voltages for a fixed time, and the extrapolated instantaneous tail current is measured after repolarizing the membrane to −100 mV (Fig. 3). The plot of the current magnitude *vs.* voltage was described by a Boltzmann equation with a $V_{½}$ of near −20 mV. Treatment of the oocyte with TRH shifted the $V_{½}$ value by 18 ± 2 mV ($n = 3$) in the depolarizing direction.

The time dependence of HERG fast inactivation from the open state was determined by a dual-pulse protocol, looking at the single exponential decline in current along the second depolarization pulse (see Methods). As shown in Fig. 4, the rate of inactivation was voltage dependent (see also Schönherr & Heinemann, 1996; Smith *et al.* 1996; Spector *et al.* 1996*b*; Wang *et al.* 1996). However, it remained unaltered at all voltages tested after challenging the cell with TRH. Failure to detect any change in the time course of inactivation was not due to a lack of TRH effects in this particular cell. As shown in the inset of Fig. 4, a clear acceleration of deactivation was induced in the same oocyte by the TRH treatment.

Altogether, these results indicate that activation of the TRH receptor modifies the activation and deactivation gating properties of HERG channels. Such a modification causes an acceleration of channel deactivation, a slower activation, and a shift in the voltage dependence of activation towards more positive voltages. However, neither the inactivation transitions from the open state nor the inactivation recovery process are influenced by treating the cells with TRH.

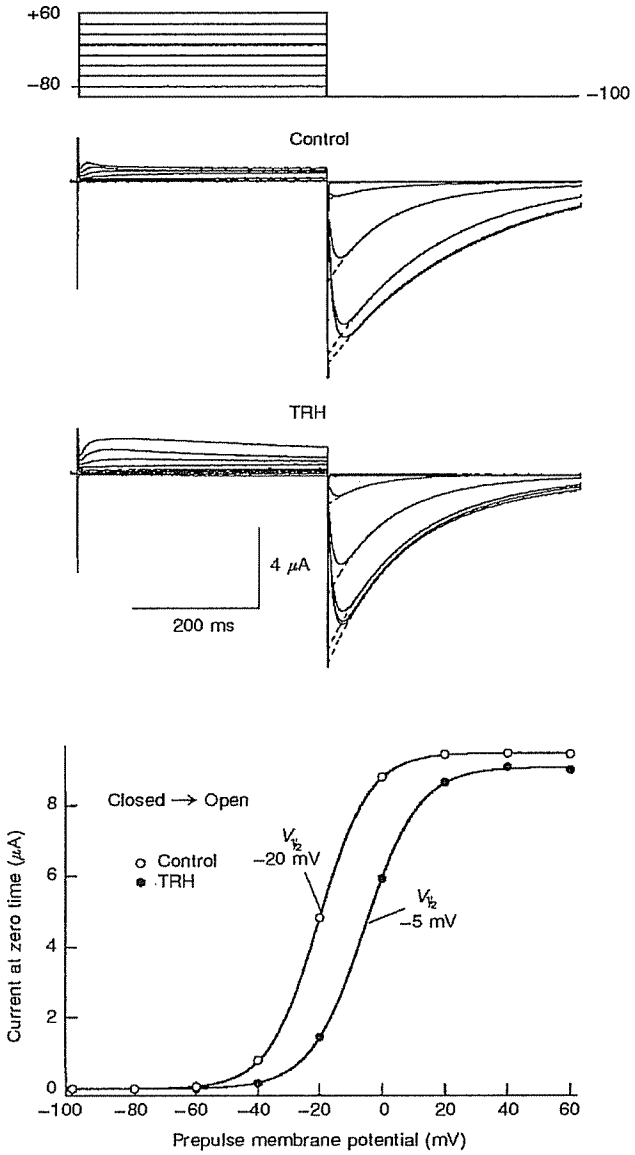

Figure 3. Effect of TRH on HERG channel voltage dependence of activation

Voltage dependence of activation was studied in the absence (control) or the presence of 1 μM TRH by varying the magnitude of a 400 ms prepulse according to the voltage protocol shown at the top. Test pulses were applied once every 20 s. When the effect of TRH was tested, data collection started 2 min after challenging the cell with the neuropeptide. The magnitude of the instantaneous tail current at −100 mV was determined by fitting an exponential to the decaying portion of the tail as shown superimposed on the tail currents, and extrapolating the current to the moment the depolarizing pulse was ended. The continuous lines in the lower panel correspond to Boltzmann curves: $h(V) = I_{max}(1/(1 + \exp(V - V_{½})/k))$, which best fitted to the data with $V_{½}$ of −20 and −5 mV, I_{max} of 9·4 and 9·1 μA, and k values of −8·1 and −8·7 for control and TRH, respectively.

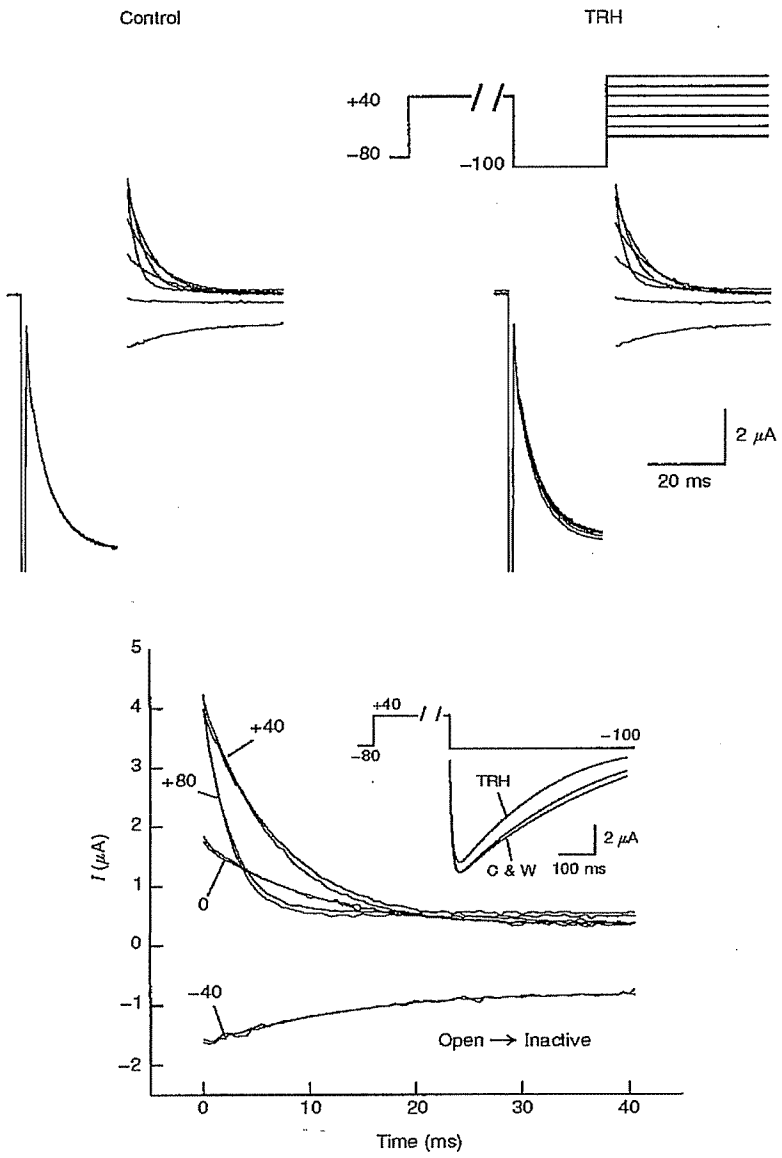

Figure 4. Lack of TRH effects on HERG channel inactivation kinetics

Onset of fast inactivation was studied using the voltage protocol shown at the top. HERG was activated and inactivated with a 400 ms prepulse to +40 mV. A 20 ms pulse to −100 mV was used to recover the channels from inactivation, followed by a test pulse to different voltages to re-inactivate the channels. Test pulses were applied once every 20 s. The decaying portion of the current during the test pulse is shown starting 2·5 ms after the hyperpolarizing pulse to −100 mV was ended. Membrane currents recorded at the end of the depolarization prepulse and along the 20 ms hyperpolarization pulse are also shown. The two families of current traces shown at the top were obtained in the absence (control) or starting 2 min after challenging the cell with 1 μM TRH. Inactivating decaying currents along the test pulses to −40, 0, +40 and +80 mV both in the absence or in the presence of TRH are shown superimposed in the lower panel. Note the almost exact correspondence of both currents at all tested voltages. TRH effects on inactivation kinetics were not observed even though the hormone induced a clear acceleration of the deactivation rate in the same oocyte, as shown in the inset. In this case, inward tail currents during long hyperpolarizations to −100 mV are shown. Currents were recorded either before (C), 90 s after start the TRH treatment (TRH), or after 10 min of hormone washout (W).

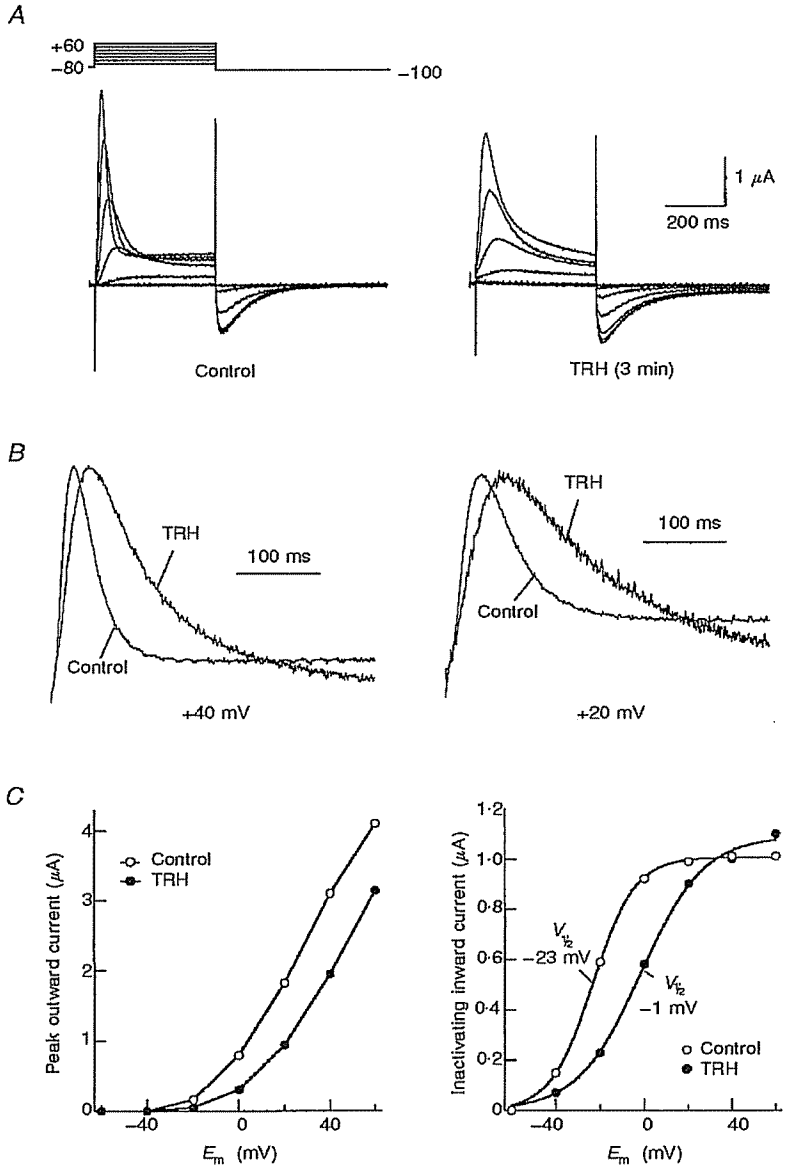

Figure 5. Effect of TRH on activation gating of HERG channels in Cs^+-containing extracellular solutions

A, comparison of membrane currents elicited by membrane depolarization before (control) or 3 min after starting the treatment with 1 μM TRH (TRH). Pulses (400 ms) were applied to test potentials of −60 to +60 mV in 20 mV steps from a holding potential of −80 mV. Test pulses were applied once every 20 s. Current traces shown have been subtracted for leak. Extracellular solutions containing Cs^+ instead of K^+ were used. *B*, slower activation rates are induced by TRH on outward K^+currents studied in Cs^+-containing extracellular solutions. Outward currents obtained in the absence (control) or the presence of TRH are shown for depolarizing pulses to +40 (left) or +20 mV (right). For comparison, currents were normalized to peak. *C*, left, effect of TRH on the magnitude of outward currents as a function of membrane potential. Outward current magnitudes at the peak are plotted *vs.* test pulse potential (E_m) for the currents shown in *A*. The data points are connected by straight lines. Right, effect of TRH on the magnitude of Cs^+ inward tail currents as a function of membrane potential. The magnitude of the tail currents is plotted *vs.* test pulse potential for the currents shown in *A*. The continuous lines correspond to Boltzmann curves that best fitted the data with $V_{½}$ of −23 and −1 mV, I_{max} of 1·0 and 1·1 μA, and k values of −9·5 and −14·2 for control and TRH, respectively.

TRH-mediated modification of outward current kinetic parameters in Cs^+-containing solutions

As indicated above, the superposition of quite unusual fast inactivation and slow activation kinetics makes it necessary to perform an indirect estimation of activation gating parameters through measurements of the recovery from open (O) plus inactive (I) states along the tail currents. However, as discussed below, this estimation can be biased by occurrence of inactivation directly from a closed state, that deviates from a sequential model for channel behaviour. To obtain a more direct demonstration of alterations in the activation rate, we took advantage from the fact that replacement of extracellular K^+ by Cs^+ causes a marked increase in outward currents (Schönherr & Heinemann, 1996). Although a direct effect of Cs^+ replacement on closed (C) to O transitions cannot be completely excluded, we found that this outward current increase can be induced, at least in part, by a twofold slower rate of inactivation in the presence of external Cs^+ (data not shown). In any case, we compared the speed of channel activation before and after challenging the oocytes with TRH, by measuring the time necessary to reach the peak of the outward current in extracellular Cs^+. Figure 5*A* and *B* shows that, as predicted from the results obtained in K^+-containing extracellular solutions, the activation time course was slowed by TRH at all voltages between −20 and 60 mV (see also Table 1). Furthermore, the neuropeptide caused a shift of near 20 mV in the voltage dependence for activation also in Cs^+-containing solutions (Fig. 5*C*). Again, the kinetic alterations were not related to a modification of inactivation rates, since the time course of the O to I transitions remained unaltered after challenging the oocytes with TRH in Cs^+-containing solutions (not shown). These results demonstrate in a more direct way that activation of the TRH-R is indeed able to slow down the activation kinetics of the HERG channels.

The effects of TRH on activation and deactivation kinetics are mimicked by direct activation of PKC with β-phorbol 12-myristate, 13-acetate (PMA)

TRH-R activation of PLC leads to formation of diacylglycerol and activation of PKC, a Ca^{2+}- and lipid-dependent kinase. To examine whether PKC modulates HERG channels we exposed oocytes expressing these channels to PMA, a pharmacologically specific activator of PKC. As with TRH, addition of PMA (100 nM) caused an acceleration of deactivation in a time-dependent manner. Although the PMA effect developed more slowly than that of TRH, 5–10 min after starting the treatment the time constant of tail current decay at −100 mV was reduced by 20% and 50–60% with 10 and 100 nM PMA, respectively (Fig. 6*A*). The effect of PMA was due to its action activating PKC. Thus pre-incubation of the oocytes with 3 μM GF109203X (a highly specific, non-toxic inhibitor of PKC; Crespo *et al.* 1994) for 1–3 h abolished the effect of 10 nM PMA and significantly reduced that of 100 nM PMA. Almost complete inhibition of the effect elicited by 100 nM PMA was achieved by raising the GF109203X concentration to 10 μM and the incubation time to 2–6 h (Fig. 6*A*). It is interesting that the effects of PMA were not accompanied by any modification of oocyte Ca^{2+}-dependent Cl^- currents. This further supports our conclusion that the alterations in current kinetics induced by TRH are not related to any effect of the hormone on Ca^{2+}-dependent Cl^- conductance.

To extend further the parallel between the actions of PMA and TRH, we also studied the effects of the PKC activator on activation and inactivation kinetic parameters. The rate of recovery from inactivation remained largely unaltered in the presence of PMA (not shown). This indicates that, as for TRH, the effects on tail current kinetics caused by direct activation of PKC with PMA are predominantly manifested in an acceleration of deactivation.

The results shown in Fig. 6*B* indicate that the activation gating transitions are slowed by challenging the cell with PMA. The time course of transitions from C to O plus I states was initially studied by varying the duration of the depolarization pulses and looking at the magnitude of the tail currents along a subsequent hyperpolarization (see above). In this case, a progressive increase in the activation time constant was obtained in the presence of PMA, leading to a twofold enhancement after 10 min of PMA treatment. This increase was significantly reduced in oocytes incubated with GF109203X (not shown). For a more direct demonstration that the C to O transition is slowed by PMA, we compared the speed of the outward current onset in Cs^+-containing extracellular solutions. As shown in Fig. 6*B*, the activation time course of the current was clearly slowed by treatment with 100 nM PMA for 10 min. Furthermore, as with TRH, a nearly 20 mV shift in the depolarizing direction was also induced by PMA on the voltage dependence of activation in Cs^+-containing solutions. Interestingly, the increase in the time to reach the peak of the outward current was paralleled by a 30% reduction in the time constant of the tail current decay (inset, Fig. 6*B*). This indicates that the acceleration of the deactivation process by PKC activation is exerted regardless of the nature of the permeant ion carrying the inward current: Cs^+ instead of K^+ in the experiment shown in Fig. 6*B*.

The clear parallel between the effects of TRH and PMA on kinetic properties of HERG channels is strengthened by the lack of PMA effects on fast inactivation. As shown in Fig. 6*C*, the time course of inactivation from the open state remained unaltered in the presence of PMA, although the tail inward currents were ostensibly modified by the drug in the same cell (Fig. 6*C*, inset). Altogether, this suggests that modulation of HERG channel gating properties by the PLC-activating TRH-R is probably achieved through a pathway at least partially dependent on PKC activation.

Effects of protein kinase inhibitors on regulation of HERG deactivation by TRH

As a final demonstration that a PKC-dependent mechanism participates in regulation of HERG gating by TRH, the effect of the neuropeptide was studied in the presence of

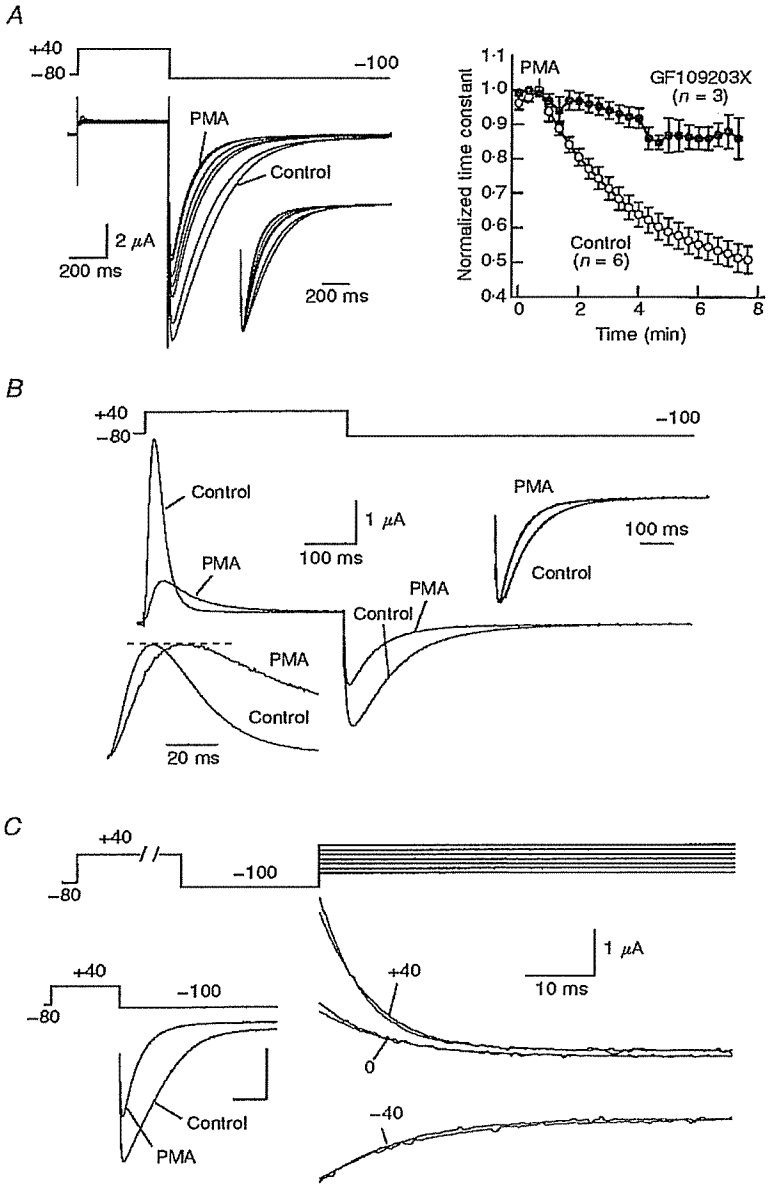

Figure 6. Effect of PMA on HERG channel kinetics

A, acceleration of deactivation rates in response to PMA. Superimposed current traces in response to the indicated potential protocol are shown on the left. Test pulses were delivered once every 20 s to an oocyte bathed in extracellular high-K^+ medium. Only traces obtained before (control) and every minute after the start of 100 nM PMA addition are shown. Tail currents normalized to peak are shown in the inset for a better comparison of current kinetics. Note the correspondence of the initial rising phase of the tails. Averaged values of deactivation time constants for 6 control oocytes and 3 oocytes preincubated with 10 μM GF109203X for 2–3 h from the same donor are shown on the right. Similar results were obtained in 4 additional experiments with control oocytes (19 oocytes from 5 frogs) and 2 additional experiments with oocytes treated with GF109203X (11 oocytes from 3 frogs). Time constants were normalized to that measured at the time of PMA addition. *B*, slow-down of outward HERG current kinetics by PMA in Cs^+-containing extracellular solutions. Membrane currents obtained with the voltage protocol shown at the top in the absence (control) or 5 min after addition of 100 nM PMA are shown superimposed. Outward currents normalized to peak are shown in the inset on the lower left for comparison. Normalized tail currents are also shown (inset, upper right). *C*, lack of PMA effect on HERG channel inactivation kinetics. Onset of fast inactivation was studied using an oocyte bathed in extracellular high-K^+ medium, using the double-pulse protocol shown at the top (see legend to Fig. 5). Inactivating decaying currents along the test pulses to −40, 0 and +40 mV are shown both in the absence or presence of 100 nM PMA for 10 min. Note that currents in both conditions are practically indistinguishable. However, a clear effect of PMA on deactivation rates was observed in the same oocyte, as shown by a marked acceleration of hyperpolarization-induced tail current decay (inset, lower left corner).

GF109203X, under conditions known to eliminate the PKC-dependent PMA-mediated modification of HERG kinetics (see above). As shown in Fig. 7*A*, the acceleration of HERG tail current decay in response to TRH was abolished in oocytes treated with GF109203X. In this case, a limited but persistent increase of deactivation rates was usually induced by the hormone, which paralleled the reversion of the TRH effect in control untreated oocytes upon hormone washout. The reason for this behaviour is not known. However, it seems to be a consequence of the TRH treatment, since it was not observed upon addition of PMA to oocytes incubated with GF109203X.

Besides an activating effect of TRH on PKC-dependent protein phosphorylation, it could also be expected that elevations of cytoplasmic Ca^{2+} concentration after liberation of the cation from intracellular stores would lead to activation of Ca^{2+}-dependent enzymatic activities such as Ca^{2+}–calmodulin protein kinase II. This prompted us to check whether this Ca^{2+}-dependent phosphorylating activity is involved in the effects of the neuropeptide. As shown in Fig. 7*B*, the TRH-dependent acceleration of deactivation kinetics remained unaltered in oocytes treated with the highly specific Ca^{2+}–calmodulin protein kinase II inhibitor KN-62. Finally, a similar acceleration of tail current decay was also induced by TRH in the presence of the tyrosine kinase inhibitor genistein (Fig. 7*C*). This indicates that, unlike previous results obtained with Kv1.2 K^+ channels (Huang *et al.* 1993), the PKC-dependent effects on HERG channel gating are not exerted through a transduction cascade, which implicates a tyrosine kinase that directly phosphorylates the channel protein.

DISCUSSION

In this study we show that the gating of HERG channels expressed in oocytes is modulated by the G protein-coupled TRH-R through a pathway involving PLC stimulation and activation of PKC. By co-expressing the cloned TRH-R and HERG channel in the oocyte model system, our results demonstrate that activation of TRH-R accelerates channel

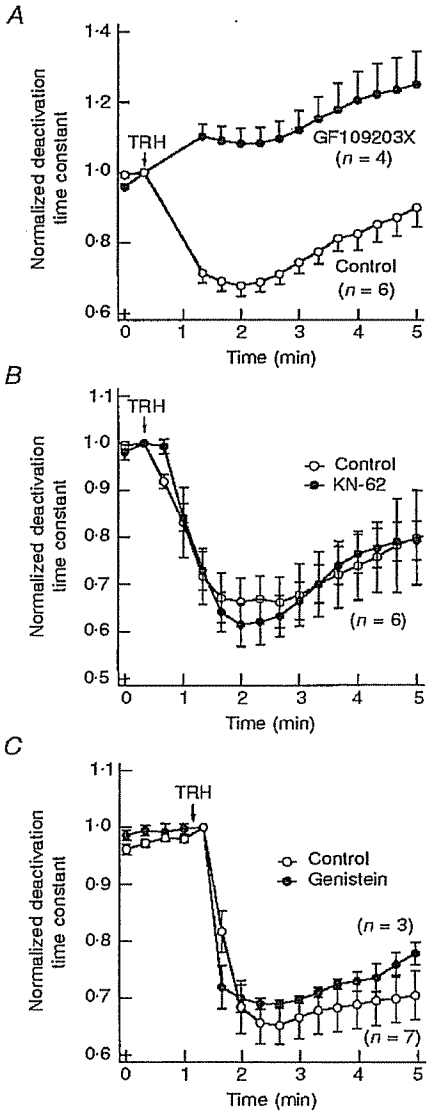

Figure 7. Effect of different protein kinase inhibitors on TRH-induced modification of HERG deactivation kinetics

A, blockade of the TRH effect by the PKC inhibitor GF109203X. Deactivation time constants normalized to that measured at the moment of TRH addition are shown for untreated oocytes (control) or oocytes incubated for 4–6 h with 10 μM GF109203X. Test pulses were delivered to the cells every 20 s. Time constant values for the two pulses following introduction of TRH into the chamber have been deleted for clarity. The start of a 30 s perfusion with medium containing 1 μM TRH is indicated. *B*, TRH-induced effects in the presence of the Ca^{2+}–calmodulin protein kinase II inhibitor KN-62. Variations in the deactivation time constant for untreated oocytes (control) and oocytes incubated for 1–3 h with 20 μM KN-62 are shown. The start of a 30 s perfusion with medium containing 1 μM TRH is indicated at the top of the graph. *C*, TRH-induced effects in the presence of the tyrosine kinase inhibitor genistein. Normalized deactivation time constant values are shown for oocytes injected with a micropipette filled with 100 μM genistein (final intracellular concentration approximately 5 μM genistein) or vehicle (control) 30 min before start of HERG current recordings. The start of perfusion with 1 μM TRH is indicated. In this case, the hormone remained present up to the end of the experiments.

deactivation and slows down the activation rate. These effects are specifically exerted on voltage-dependent conformational changes leading to channel activation and closure, since neither the inactivation transitions from the open state nor the inactivation recovery rates are modified after challenging the oocytes with TRH.

PKC is activated physiologically by the co-ordinate action of DAG and Ca^{2+} in the presence of phosphatidylserine. Our data indicate that the PKC branch of the PLC signalling cascade participates in modulation of HERG channel gating. Thus the effect of TRH was mimicked by direct pharmacological activation of PKC with PMA. Interestingly, as with TRH, only channel activation and deactivation parameters, but not the onset or recovery rates of inactivation were modified by PMA, emphasizing the parallel between the actions of both regulators. Furthermore, the TRH effect was antagonized by GF109203X, a highly specific inhibitor of PKC that also abolished the PMA-dependent regulation of the channels, but not by the inhibitors of tyrosine kinases and Ca^{2+}–calmodulin serine/threonine kinase, genistein and KN-62. However, it is important to note that our data do not allow us to distinguish between a direct phosphorylating action of PKC on the channel (or an auxiliary subunit associated to it), or an indirect effect of PKC on an early step of the transduction cascade.

A remarkable characteristic of HERG channels is the presence of a rapid and voltage-dependent inactivation process that reduces conductance at positive voltages and strongly limits the level of outward current after depolarizing the membrane. This precludes the accurate estimation of activation kinetic parameters by looking at the time-dependent development of outward currents. Assuming a sequential model for channel behaviour ($C1 \ldots Cn \rightleftharpoons O \rightleftharpoons I$), the activation transitions can be characterized by using an envelope of tail currents protocol (see Methods). However, this would yield an estimation only of the rate at which the channels leave the closed state either through the open state or directly to the inactive state, if inactivation occurs directly from a closed state with a similar time dependence. Such a direct inactivation pathway has been previously observed for other structurally related voltage-dependent K^+ channels such as Kv1.3 (Marom & Levitan, 1994) and Kv1.4 (F. Barros and P. de la Peña, unpublished data). It could also constitute a relevant pathway for HERG, in which inactivation seems to develop more quickly than activation (Spector *et al.* 1996*b*). Furthermore, this would complicate the interpretation of the regulatory effects of TRH and PMA on channel kinetics. Nevertheless, our results strongly suggest that both agents certainly induce a slowing of the activation time course besides an acceleration of deactivation. Thus the delaying actions of TRH and PMA were also demonstrated in a more direct way by looking at the markedly increased outward currents elicited in extracellular solutions in which K^+ had been replaced by Cs^+. It would be interesting to know whether similar effects to those reported here can be obtained with channel variants from which inactivation has been removed by site-directed mutagenesis (Schönherr & Heinemann, 1996; Smith *et al.* 1996).

The more generalized model for K^+ channel behaviour (Zagotta & Aldrich, 1990; Hoshi *et al.* 1994) involves a voltage-dependent channel gating. Subsequently, many channels of the *Shaker* superfamily undergo inactivation by N-type or C-type mechanisms. The inactivation is generally regarded as a voltage-insensitive process in which any apparent voltage dependence arises from direct coupling to activation. In such a theoretical background it could be possible that, as shown here for HERG channels, phosphorylation may act by altering the voltage-dependent gating without significant modifications of the intrinsically voltage-independent transitions leading to inactivation. However, the fast time-dependent inactivation of HERG is strongly voltage dependent, both in the hyperpolarized and depolarized ranges of membrane potentials. Such a voltage dependence does not seem to arise from activation (Wang *et al.* 1996, 1997). Thus if phosphorylation of HERG exclusively disrupts the electrostatic interactions that control gating (Perozo & Bezanilla, 1990), it would be necessary that the activation and the inactivation apparatus lie in a different protein domain. Further knowledge of the structure of this channel would be necessary to prove such a hypothesis.

Previous work with Kv3.4 channels showed a reduction of channel inactivation through a direct PKC-dependent phosphorylation of sites located at the N-terminus (Covarrubias *et al.* 1994). The phosphorylation of the Kv3.4 inactivation gate modulates N-type inactivation in this channel. However, a C-type and not an N-type mechanism governs the inactivation of HERG (Schönherr & Heinemann, 1996; Smith *et al.* 1996; but see also Wang *et al.* 1996, 1997). Furthermore, the N-terminal domain has been implicated in the activation and deactivation of HERG, but not in the inactivation mechanism (Schönherr & Heinemann, 1996; Spector *et al.* 1996*b*). Thus the specificity of the TRH and PMA effects on activation and deactivation parameters would be coherent with the idea that a direct phosphorylation of the N-terminal domain by PKC regulates the gating properties of HERG channels. This is also supported by the marked acceleration of channel deactivation caused by deletion of the HERG cytoplasmic amino terminus without a severe change in inactivation kinetics (Schönherr & Heinemann, 1996; Spector *et al.* 1996*b*). As for other related channels, a net positive charge is present within the first thirty amino acids of HERG. It remains to be established whether phosphorylation of this or a nearby domain may disrupt some electrostatic interactions affecting channel gating.

It is important to note that although it is possible that PKC-dependent phosphorylation of the HERG N-terminal domain participates in channel regulation, other possibilities also exist. Thus phosphorylation of a C-terminal site by protein kinase A modifies interaction of the *Shaker* N-terminal domain with its receptor site(s) in the core of the protein (Drain *et al.* 1994). Similarly, phosphorylation of the 'chain'

connecting the N-terminal inactivation ball of Kv1.4 with the core of the channel protein seems to regulate the interactions between the inactivation particle and its receptor site (Roeper *et al.* 1997). Clearly, further experiments are necessary to define the specific site(s) phosphorylated by PKC and its relevance in channel function.

The importance of HERG currents in regulating cardiac beat and action potential duration has been largely recognized. More recently, HERG (Curran *et al.* 1995; Sanguinetti *et al.* 1995; Spector *et al.* 1996*a*) and minK/KvLQT1 (Chouabe *et al.* 1997) channel malfunction has been implicated in some types of cardiac arrhythmia. The physiological relevance of the regulatory mechanism described here is twofold. Firstly, it is unlikely that TRH receptors are expressed in cardiac tissues, but the participation of a PKC-dependent pathway in channel regulation makes it generally applicable to other PLC-coupled receptors. Thus analogous effects to those caused by TRH-R activation are observed in response to serotonin when oocytes co-expressing HERG and serotonin-1c receptors are used (F. Barros and P. de la Peña, unpublished data). Since different transducers are used to couple TRH and serotonin-1c receptors to oocyte PLC activation (Quick *et al.* 1994; de la Peña *et al.* 1995), these results further emphasize the general significance of the mechanism described here for regulation of HERG channels, and subsequently of cardiac function. Interestingly, PMA-induced phosphorylation of minK by PKC also inhibits the cardiac slow delayed rectifier current I_{Ks} and shifts its activation voltage dependence to more depolarized voltages (Busch *et al.* 1992). Thus our results implicate for the first time the activation of physiologically active hormone and neurotransmitter receptors coupled to PLC in the concerted regulation of the two K^+ current components that repolarize the cardiac action potential: I_{Kr}, contributed by HERG (Sanguinetti *et al.* 1995; Trudeau *et al.* 1995), and I_{Ks}, contributed by minK/KvLQT1 (Barhanin *et al.* 1996; Sanguinetti *et al.* 1996). Secondly, although obtained in the oocyte model system, our results can provide an answer to a long-standing question about how TRH modulates neurosecretion along the named second phase of hormone action. Inwardly rectifying K^+ currents constitute an important TRH target for control of electrical activity in adenohypophysial cells (Barros *et al.* 1994, 1997). Recent kinetic and pharmacological evidence indicates that a HERG-like channel is the cause of such TRH-regulated currents (Barros *et al.* 1997). Furthermore, a phosphorylation mechanism is involved in both inwardly rectifying current inhibition and action potential frequency increases in response to TRH (Barros *et al.* 1992, 1993; Delgado *et al.* 1992). It should be noted, however, that the participation of PKC in electrical responses to TRH has remained controversial (Barros *et al.* 1992, 1993, 1994), and an unknown protein kinase distinct from PKC and protein kinase A has been implicated in regulation of GH_3 cell HERG-like currents by TRH (Barros *et al.* 1993). Whether this is due to differences in some molecular characteristics between HERG channels and their adenohypophysial counterparts remains to be established. It would be interesting to know if a mechanism similar to that reported here modulates the function of the rat erg channel recently isolated from GH_3/B6 cells (contributed by B. Engeland and J. Ludwig, under accession number Z96106).

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Role of Phospholipase C-β in the Modulation of Epithelial Tight Junction Permeability

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ABSTRACT

The results presented in this study establish an association between phospholipase C-β (PLC-β) and tight junction permeability across Madin-Darby canine kidney (MDCK) cell monolayers, an in vitro model for epithelial tissue. These results further show that PLC-β modulates tight junction permeability by affecting actin filament organization. Hexadecylphosphocholine (HPC) inhibited PLC-β and increased tight junction permeability in MDCK cells. Interestingly, the analogs of HPC, a series of alkylphosphocholines containing various lengths of linear alkyl chains, inhibited PLC-β and increased tight junction permeability with a wide range of potency. The potency of alkylphosphocholines as enhancers of tight junction permeability significantly correlated ($p < 0.05$) with their potency as PLC-β inhibitors. U73122, a steroid derivative that is structurally unrelated to alkylphosphocholines, inhibited PLC-β and increased tight junction permeability with potencies that fit into the correlation observed for the alkylphosphocholine series. U73122 and HPC induced disorganization of actin filaments in MDCK cell monolayers. The potencies to cause disorganization of actin filaments were consistent with the potencies of these agents as inhibitors of PLC-β and enhancers of tight junction permeability. Furthermore, ATP, an activator of PLC-β, attenuated U73122-induced increase in tight junction permeability as well as disorganization of actin filaments. These results provide strong evidence that PLC-β inhibition leads to increased tight junction permeability across MDCK cell monolayers through disorganization of actin filaments.

Epithelial and endothelial cellular sheets act as barriers to separate the body from the external environment, and maintain distinct compartments within the multicellular organism. An important component of this barrier is the presence of intercellular junctions, including the zona occludens (tight junctions), which restricts the movement of molecules across cell monolayers (Diamond, 1977; Schneeberger and Lynch, 1992; Anderson and Van Itallie, 1995). Restriction of ion movement imposed by tight junctions across the epithelium gives rise to transepithelial electrical resistance (TEER), which is often used as an index of tight junction integrity. This barrier function of the tight junction is not static. Thus, many hydrophilic nutrients easily cross the epithelium through the paracellular pathway (Ballard et al., 1995), and evidence is emerging that epithelial cells regulate the movement of molecules across cell monolayers by modulating the permeability across the tight junctions (Ward et al., 2000).

The tight junction is a complex structure composed of both transmembrane and cytosolic proteins (Denker and Nigam, 1998; Fanning et al., 1999). Many of these proteins (e.g., occludin, ZO-1, ZO-2, and ZO-3) have phosphorylation sites, suggesting that these proteins are possible endpoints of various signal cascades (Anderson et al., 1988; Sakakibara et al., 1997). Currently, many signaling pathways involving tyrosine kinases, calcium, and protein kinase C (PKC) have been implicated in the regulation of tight junction permeability (Anderson and Van Itallie, 1995; Tai et al., 1996; Collares-Buzato et al., 1998; Mullin et al., 1998). The molecular mechanisms associated with this regulation, however, have not been elucidated.

Phospholipase C (PLC), an important regulatory enzyme, catalyzes hydrolysis of phosphatidylinositol-4,5-bisphosphate into inositol triphosphate and diacylglycerol in response to the stimulation of a variety of receptors, e.g., stimulation of $P2Y_2$ receptors by ATP (Nicholas et al., 1996). Activity of different families of PLC is regulated through

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ABBREVIATIONS: TEER, transepithelial electrical resistance; PKC, protein kinase C; PLC, phospholipase C; EGF, epidermal growth factor; MDCK, Madin-Darby canine kidney; C10, C12, C14, C18, C20, alkylphosphocholines containing indicated numbers of carbons in the alkyl chain; HPC, hexadecylphosphocholine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

different receptor-mediated pathways. For example, the activities of PLC-β and PLC-γ are regulated through G-protein-coupled receptors (e.g., purinergic receptor) and receptor tyrosine kinases (e.g., epidermal growth factor receptor), respectively (Katan, 1998). A PLC-dependent pathway has been implicated in the assembly of the tight junction (Balda et al., 1991; Cereijido et al., 1993a,b; Emori et al., 1994; Thackeray et al., 1998; Wang et al., 1998; Fleming et al., 2000), particularly during development, although evidence for its role in the function of mature tight junctions is ambiguous at best. For example, it has been reported that a medium-chain fatty acid causes increased tight junction permeability via activation of PLC, whereas its close structural analog does not act via PLC (Lindmark et al., 1998). Hexadecylphosphocholine (HPC), an inhibitor of PLC (Pawelczyk and Lowenstein, 1993; Berkovic et al., 1996), has structural features (i.e., a long alkyl chain, and a zwitterionic functionality) that have been previously identified to cause an increase in tight junction permeability (Liu et al., 1999a). HPC was found to be a potent enhancer of tight junction permeability across Madin-Darby canine kidney (MDCK) cell monolayers and a potent inhibitor of PLC in these cells. The studies described here expand on this initial finding and demonstrate that analogs of HPC, containing alkyl chains of different lengths, exhibit a wide range of potencies as 1) inhibitors of the PLC isozyme β and 2) enhancers of tight junction permeability. Thus alkylphosphocholines serve as useful mechanistic tools in our efforts to elucidate the relationship between inhibition of PLC-β and increase in tight junction permeability across MDCK cell monolayers. Additionally, our results provide evidence that changes in PLC-β activity modulates tight junction permeability by affecting changes in organization of actin filament network.

Materials and Methods

Reagents. HEPES was obtained from Lineberger Comprehensive Cancer Center (University of North Carolina at Chapel Hill). Hanks' balanced salt solution was obtained from Mediatech (Hernford, VA). Cell culture reagents were obtained from Invitrogen (Carlsbad, CA). Transwell inserts (12 wells/plate, 3-μm pore, and 1.0 cm^2 area with polycarbonate or polyester membranes) and plates (12-well) were obtained from Costar (Cambridge, MA). [^{14}C]Mannitol and [^{3}H]myo-inositol were obtained from American Radiolabeled Chemicals (St. Louis, MO). Dodecylphosphocholine was obtained from Avanti Polar Lipids (Alabaster, AL). AG1-X8 formate columns were obtained from Bio-Rad (Hercules, CA). Phalloidin conjugated with Texas Red was obtained from Molecular Probes (Eugene, OR). All other compounds and reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Cell Culture. MDCK epithelial cell line strain II was obtained from the American Type Culture Collection (Manassas, VA) through the Lineberger Comprehensive Cancer Center. MDCK cells derived from normal proximal kidney epithelial cells of a cocker spaniel, which serve as a model for epithelial transport experiments (Cho et al., 1989), were grown to confluence on Transwell inserts with polycarbonate membranes to determine the effect of absorption enhancers on TEER and mannitol transport. The cells were grown on Transwell inserts with transparent polyester membranes for confocal studies. Each well was seeded with cells at a density of 100,000 cells/cm^2. Cells were then grown in cell medium (minimum essential medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B, and supplemented with 10% fetal bovine serum and 0.1 mM nonessential amino acids) and were maintained at 37°C under 5% CO_2. Cells were grown for 4 days during which they differentiated into epithelial cell monolayers, as evidenced by the establishment of a stable TEER between 150 and 250 $\Omega \cdot cm^2$ [EVOM Epithelial Tissue Voltohmeter (World Precision Instruments, Sarasota, FL) and an Endohm-12 electrode].

Determination of Tight Junction Permeability. TEER and mannitol flux across MDCK cell monolayers are indicators of tight junction permeability of the cell monolayer (Liu et al., 1999a,b). A decrease in TEER and/or an increase in mannitol flux across MDCK cell monolayers were used as parameters to measure the efficacy of tight junction permeability enhancers.

Determination of the Effect of Alkylphosphocholines and U73122 on TEER. Cell media were aspirated from both apical and basolateral compartments of Transwell inserts and replaced with 0.5 and 1.5 ml, respectively, of Hanks' balanced salt solution, supplemented with 10 mM HEPES, pH 7.4 (transport buffer). MDCK cell monolayers were then incubated at 37°C for 30 min, and the TEER of each cell monolayer was then measured. Experiments were initiated by replacement of transport buffer from the apical compartment of Transwell with transport buffer containing an alkylphosphocholine, U73122, or vehicle (0.5 ml). MDCK cell monolayers were incubated at 37°C, and TEER values were measured after 30 min. Data from each experiment were normalized to the response from the vehicle and were reported as the mean ± S.D. (n = 3). The effect of alkylphosphocholines and U73122 on TEER was evaluated at several concentrations, and their EC_{50} values, defined as the concentration that caused a 50% decrease in TEER with respect to the untreated control, were determined (Liu et al., 1999a,b). EC_{50} values were reported as the mean ± S.D. of three experiments, each performed in triplicate.

Determination of the Effect of Alkylphosphocholines and U73122 on Mannitol Transport. Cell media were aspirated from apical and basolateral compartments of Transwell and replaced with transport buffer. MDCK cell monolayers were then incubated at 37°C for 30 min. The integrity of tight junctions of the cell monolayer was monitored by measurement of TEER prior to the experiment (150–250 $\Omega \cdot cm^2$). Transport experiments were initiated by replacing the transport buffer in the apical compartment with 0.5 ml of transport buffer containing an alkylphosphocholine, U73122, or vehicle, and [^{14}C]mannitol (25 μM, 55 mCi/mmol). Transport rates were monitored by quantifying the amount of [^{14}C]mannitol accumulated (Packard Tri Carb 4000 Series liquid scintillation spectrophotometer) in the basolateral side (1.5 ml) between 30- and 60-min intervals after initiating the treatment. All transport experiments were conducted under sink conditions (less than 10% of the total amount of [^{14}C]mannitol was present on the basolateral side at any given time). The flux of [^{14}C]mannitol in the presence of an alkylphosphocholine or U73122 was normalized to that in the vehicle-treated cells and was reported as the mean ± S.D. (n = 3). EC_{10x}, defined as the concentration of an alkylphosphocholine or U73122 that causes a 10-fold increase in mannitol flux with respect to the vehicle-treated control (Liu et al., 1999a,b), was determined for each enhancer of tight junction permeability, and was reported as the mean ± S.D. of three experiments, each performed in triplicate.

Determination of PLC-β Activity by Cellular Assay. The activity of PLC-β in MDCK cells, transfected with $P2Y_2$ receptors, was determined by an adaptation of a previously published method (Schachter et al., 1997). MDCK cells, transfected with $P2Y_2$ receptors, were seeded at 400,000 cells/cm^2 and subsequently cultured for 4 days. The cell monolayers were then labeled with [^{3}H]myo-inositol (1.6 μCi/well in 0.4 ml of inositol-free medium) for 24 h at 37°C. An alkylphosphocholine or U73122 was added at different concentrations to the medium, and the cells were incubated for 30 min at 37°C. Assays were initiated by immediately supplementing the cells with 100 μl of 250 mM HEPES (pH 7.3), containing 100 mM LiCl, with ATP (final concentration, 100 μM). The cells were then incubated at 37°C for 15 min to allow accumulation of [^{3}H]inositol phosphates. Incubations were terminated by aspiration of the medium and addition of 1 ml of boiling 10 mM EDTA (pH 8.0). The supernatant was

applied to AG1-X8 formate columns for chromatographic isolation of [^{3}H]inositol phosphates (Berridge et al., 1983). The amount of [^{3}H]-inositol phosphates was measured by liquid scintillation counting in a Packard Tri Carb 4000 series spectrophotometer. Data from each experiment were normalized to the response observed with 100 μM ATP alone and were reported as the mean ± S.D. (n = 3). $IC_{50(PLC)}$, defined as the concentration of an alkylphosphocholine or U73122 that causes a 50% decrease in ATP-stimulated PLC-β activity (accumulation of [^{3}H]inositol phosphates), was determined and reported as the mean ± S.D. of three experiments, each performed in triplicate. The cell viability upon the inhibition treatment was determined by the MTT assay (Mosmann, 1983).

Synthesis of Alkylphosphocholines. Arachidyl 2-(*N,N,N*-trimethylamino)ethyl phosphate (C20), yield 7.8%; octadecyl 2-(*N,N,N*-trimethylamino)ethyl phosphate (C18), yield 34.1%; tetradecyl 2-(*N,N,N*-trimethylamino)ethyl phosphate (C14), yield 54.0%; and decyl 2-(*N,N,N*-trimethylamino)ethyl phosphate (C10), yield 81.8% (Fig. 1) were synthesized through a two-step reaction from corresponding alcohols by previously published procedures (Hanson et al., 1982; Surles et al., 1993). The final products were characterized by mass spectrometry and nuclear magnetic resonance spectrometry.

Determination of Actin Organization with Phalloidin. Cell media were aspirated from both apical and basolateral compartments of Transwell inserts with transparent polyester membranes and replaced with transport buffer. MDCK cell monolayers were then incubated at 37°C for 30 min, and TEER of each cell monolayer was measured. Transport buffer containing different concentrations of an alkylphosphocholine, U73122, or vehicle was added to the apical side of these cells. MDCK cell monolayers were incubated at 37°C, and TEER values were measured after 30 min. Transport buffer was replaced with phalloidin conjugated with Texas Red (final concentration, 200 nM) in 3.7% formaldehyde (0.2 ml), incubated for 30 min at 4°C, and subsequently washed with transport buffer. Actin filament organization was viewed with Zeiss LSM-410 inverted laser scanning microscope (Carl Zeiss, Oberkochen, Germany), fitted with a 100× objective. Several vertical sections (x–z) of the cell monolayers were taken to define the top and bottom of this cell monolayer. En face sections (x–y) were then selected from the cortical (mid-cell) sections of the cell monolayers.

Data Analysis. Student's t test for unpaired data was used to determine significant differences ($p < 0.05$) between the mean ± S.D. from untreated and treated MDCK cell monolayers. The relation between PLC-β activity and increase in tight junction permeability was examined by linear regression analysis, and the correlation was expressed by the Pearson correlation coefficient (r).

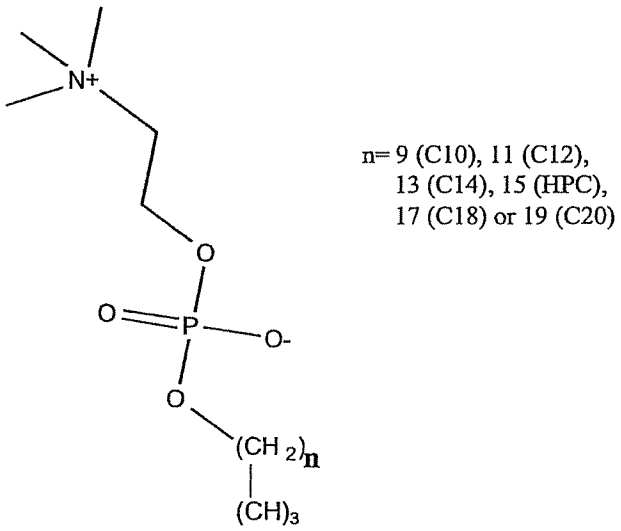

Fig. 1. Structures of alkylphosphocholines.

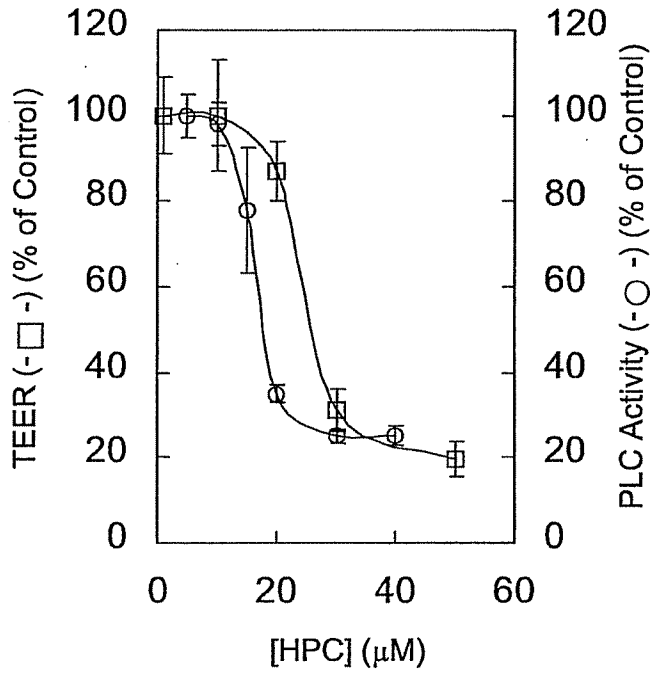

Fig. 2. The effect of HPC on TEER and ATP-stimulated PLC-β activity in MDCK cells. HPC was administered apically, and TEER was measured after 30-min incubation with HPC at 37°C. For measurement of PLC-β activity, MDCK cells transfected with $P2Y_2$ receptors were labeled with [^{3}H]myo-inositol for 24 h at 37°C. The cells were then treated with HPC for 30 min at 37°C. [^{3}H]Inositol phosphates were isolated by chromatography following treatment of the cells with ATP (100 μM) and quantified by liquid scintillation spectrometry. Data points represent mean ± S.D. (n = 3).

Results

Effect of HPC on TEER. When MDCK cell monolayers were treated with HPC (see Fig. 1 for structure) on the apical side, TEER across cell monolayers decreased as a function of time over a 60-min exposure, indicative of increased tight junction permeability. The time course of decrease in TEER was very similar to that observed with dodecylphosphocholine (C12) (Liu et al., 1999a,b). The drop in TEER when measured at a fixed time (i.e., 30 min after initial treatment) was concentration-dependent (Fig. 2). The concentration that decreased TEER by 50% (EC_{50}) was estimated to be 29 ± 5 μM (Table 1). Thus HPC appeared to be a significantly more potent enhancer of paracellular permeability than its homolog C12, which was previously tested as an enhancer of paracellular permeability in a related cell culture model of

TABLE 1
Effect of alkylphosphocholines and U73122 on TEER (EC_{50}), mannitol flux (EC_{10x}) and PLC- β activity ($IC_{50(PLC)}$) in MDCK cells
The PLC inhibition studies were conducted with MDCK cells transfected with the $P2Y_2$ receptors. Results are reported as mean ± S.D. in triplicate for three separate experiments. C10 was inactive in all three assays. Cells remained viable (>95%) over the concentration ranges used for all compounds.

	EC_{50}	EC_{10x}	$IC_{50(PLC)}$
		mM	
U73122	6 ± 2	6 ± 2	5 ± 3
HPC	29 ± 5	30 ± 8	18 ± 1
C18	59 ± 8	66 ± 11	30 ± 2
C20	71 ± 8	73 ± 1	48 ± 5
C14	44 ± 11	91 ± 11	73 ± 9
C12	733 ± 32	900 ± 100	275 ± 135

intestinal epithelium, Caco-2 cell monolayers (EC_{50} = 650 ± 50 μM) (Liu et al., 1999a).

Effect of HPC on PLC-β Activity. HPC was reported as an inhibitor of PLC in human leukemia cells (Berkovic et al., 1996). To determine whether the PLC inhibitory activity was related to its effect on TEER, it was necessary to determine whether HPC inhibited PLC activity in MDCK cell monolayers. As shown in Fig. 2, HPC decreased ATP-stimulated PLC activity (i.e., PLC-β activity; Nicholas et al., 1996) in $P2Y_2$-transfected MDCK cells. The concentration of HPC that decreased PLC-β activity by 50% [$IC_{50(PLC)}$] was estimated to be 18 ± 1 μM (Table 1). It is important to note that HPC, at concentrations that markedly inhibited ATP-stimulated PLC-β activity, had no effect on the activity of epidermal growth factor (EGF)-stimulated PLC-γ activity (Ward et al., 2002).

Effect of Alkylphosphocholines on Tight Junction Permeability across MDCK Cell Monolayers. Previous studies have shown that changes in length of the alkyl chain of glycerophosphocholines markedly affect their potencies to cause an increase in tight junction permeability (Liu et al., 1999a,b; Ouyang et al., 2002). Therefore, analogs of HPC were synthesized with systematic changes (two methylene units at a time) in the alkyl chain (Fig. 1), and their potencies to cause increase in tight junction permeability and inhibition of PLC-β activity were assessed. All compounds decreased TEER in a concentration-dependent manner, such that, at maximum concentrations, TEER was decreased to less than 20% of control values (Fig. 3A), and the EC_{50} values varied markedly (Table 1). Interestingly, small variations in the alkyl chain length of alkylphosphocholines produced significant ($p < 0.05$) changes in the EC_{50} values of these compounds. For example, HPC and C12 differed by only four methylene units in their alkyl chain, and yet their respective EC_{50} values differed by approximately 25-fold. The potency of alkylphosphocholines (EC_{50}) in this series varied over a 100-fold range, and a large drop in potency was observed between C14 and C12 (Table 1).

Alkylphosphocholines increased mannitol flux in a concen-

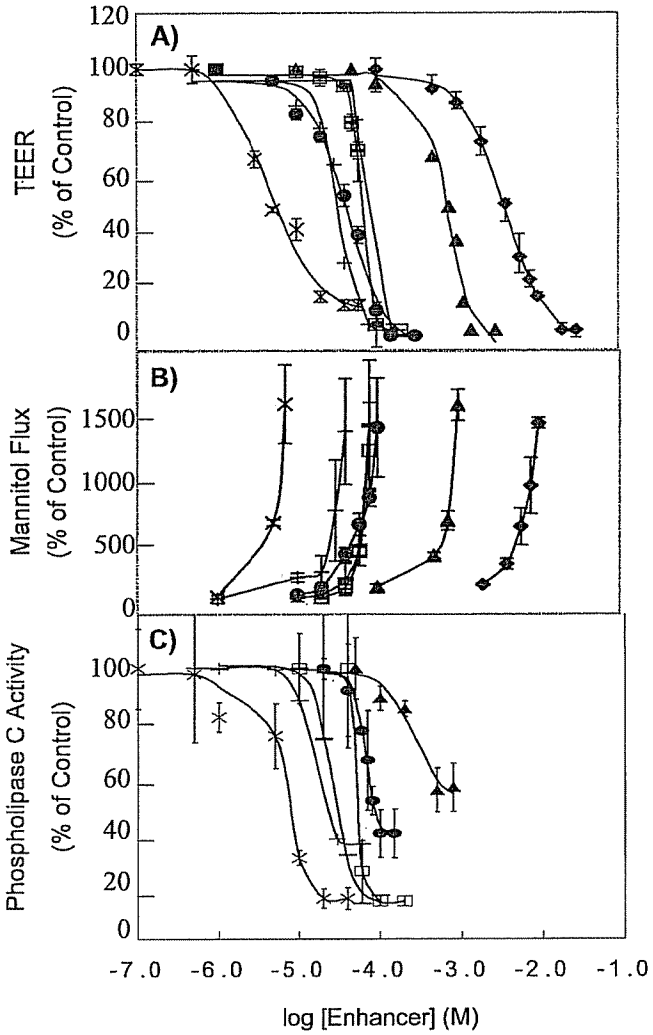

Fig. 3. The effect of alkylphosphocholines and U73122 on TEER (A), mannitol flux (B), and ATP-stimulated PLC-β activity (C) in MDCK cells. For TEER measurement the test compound was added to the apical compartment, and TEER was measured after 30-min incubation at 37°C. For measurement of mannitol flux, the test compound and [^{14}C]mannitol were added to the apical compartment, and the amount of [^{14}C]mannitol accumulated in the basolateral side (1.5 ml) during the 30- to 60-min interval after treatment with the test compound or vehicle was measured. PLC-β activity was measured in $P2Y_2$ receptor-transfected MDCK cells as described in Fig. 2. Data points represent mean ± S.D. (n = 3). Symbols that represent the compounds are: ♦, C10; ▲, C12; ●, C14; +, HPC; −, C18; □, C20; and ×, U73122.

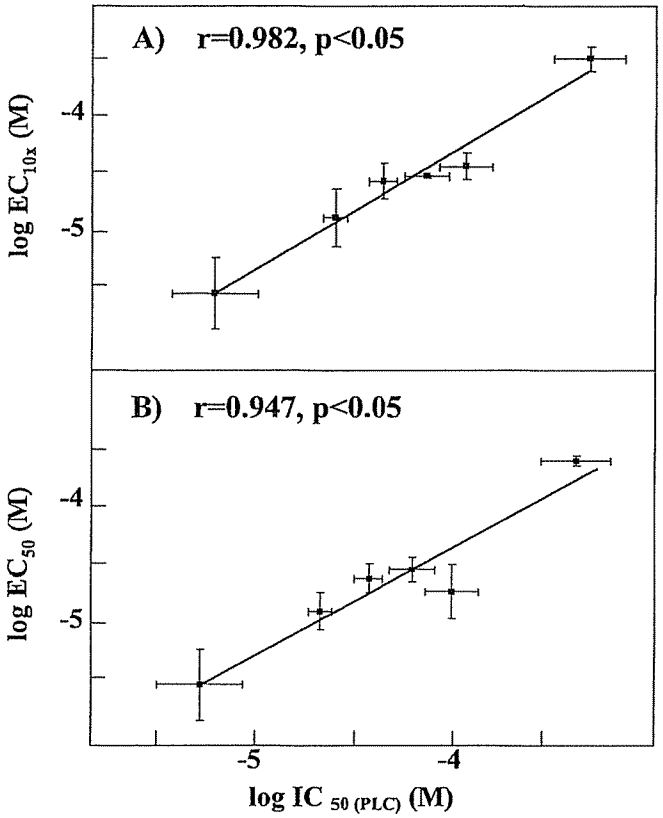

Fig. 4. Correlation between the $IC_{50(PLC)}$ and EC_{10x} values (A) and $IC_{50(PLC)}$ and EC_{50} values (B) for alkylphosphocholines and U73122. The initial correlation was observed with alkylphosphocholines only [r = 0.901 (EC_{50}), r = 0.969 (EC_{10x})], and the $IC_{50(PLC)}$, EC_{10x}, and $IC_{50(PLC)}$ values for U73122 (data enclosed in a box) were plugged in subsequently. The correlation coefficients shown in the figure include data for alkylphosphocholines and U73122. Data points represent mean ± S.D. of three experiments. The data in each experiment were obtained in triplicate.

tration-dependent manner (Fig. 3B), further confirming their ability to increase tight junction permeability. The potency of these compounds to increase tight junction permeability was expressed as EC_{10x}, defined as the concentration of compound that increased mannitol flux by 10-fold (cf. Liu et al., 1999a,b). As expected, the EC_{10x} of alkylphosphocholines (Table 1) had a good correspondence with their respective EC_{50} values. As with EC_{50}, EC_{10x} values varied over a 100-fold range with a large drop in potency between C14 and C12.

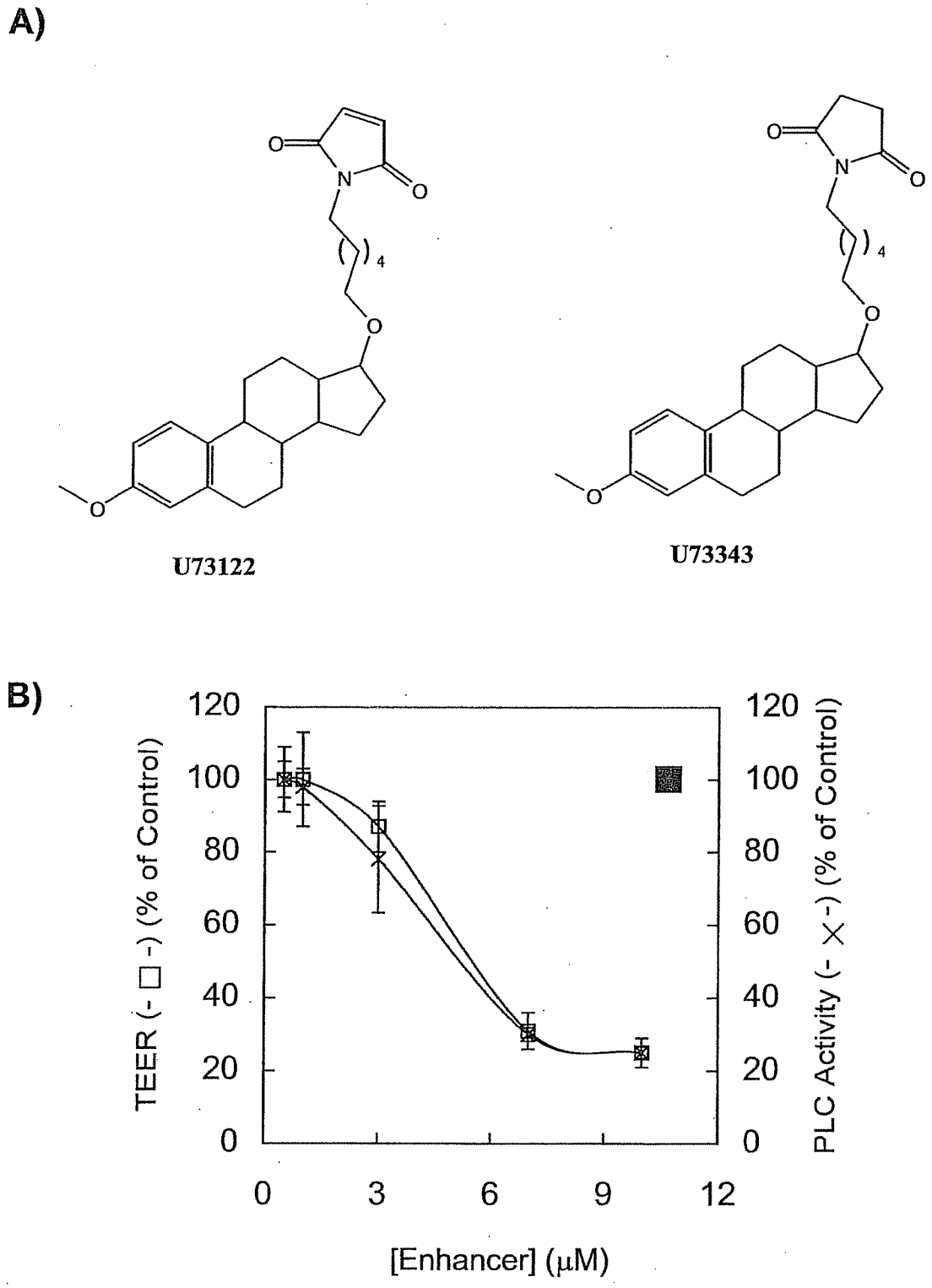

Fig. 5. Structures of the specific PLC-β inhibitor, U73122, and its inactive analog, U73343 (A), and their effect on TEER and ATP-stimulated PLC-β activity in MDCK cells (B). TEER and PLC-β activity were determined as described in Fig. 2. Data points represent mean ± S.D. (n = 3). The solid square symbol (■) represents the effect of 10 μM U73343 on TEER and PLC-β activity.

Effect of Alkylphosphocholines on PLC-β Activity in MDCK cells and Its Relationship to the Effect on Tight Junction Permeability. To determine whether PLC-β inhibition is associated with the ability of alkylphosphocholines to increase tight junction permeability in MDCK cells, the effect of these compounds on ATP-stimulated PLC-β activity (increase in inositol phosphate production) was measured as a function of concentration. As shown in Fig. 3C, many of the alkylphosphocholines tested caused inhibition of PLC-β activity. The cell viability (>95%) was not compromised, as determined by the MTT assay (Mosmann, 1983), by any of the alkylphosphocholines used in the experiments performed to determine the effect on the enzyme activity as well as on TEER and mannitol transport. The potency of alkylphosphocholines as inhibitors of PLC-β, expressed as the concentration that inhibited PLC-β activity by 50% ($IC_{50(PLC)}$), varied markedly (Fig. 3C, Table 1). For example, HPC, a potent enhancer of tight junction permeability, inhibited PLC-β with an $IC_{50(PLC)}$ of 18 ± 1 μM; whereas, the $IC_{50(PLC)}$ of C12, a weak enhancer of tight junction permeability, was 275 ± 135 μM (Table 1). The full extent of PLC-β inhibition by these compounds also varied. For example, C10, the least potent enhancer of tight junction permeability in this series, is not included in Table 1 because C10 could not reduce ATP-stimulated PLC-β activity (increase in inositol phosphate production) by 50% even at the highest concentration tested (5 mM). The full extent of PLC-β inhibition from treatment with C12 was 50% at 1 mM. This result is not surprising since C10 and C12 are the least potent enhancers of tight junction permeability in this series (Table 1). The changes in PLC inhibitory activity with changes in the chain length of alkylphosphocholines appears to be due to differences in the binding affinities of these compounds to PLC-β and not due to their different membrane permeability. This is because the precipitous drop in the PLC inhibitory activity observed between C14 and C12, and even more so between C12 and C10 (C10 is inactive), and conversely, relatively small drop of PLC inhibitory activity upon changing the chain length by six methylene groups (C14 and C20) (Table 1), is not consistent with the cell membrane permeability being a key determinant of the PLC inhibitory activity of alkylphosphocholines. The relationship between PLC-β inhibitory activity [$IC_{50(PLC)}$] of alkylphosphocholines and their potency as enhancers of tight junction permeability (EC_{50} and EC_{10x}) is depicted in Fig. 4 (also see Table 1). It appears that the potency of alkylphosphocholines is related to their potency to cause a drop in TEER and an increase in mannitol flux.

Effect of U73122 (a PLC-β Inhibitor) on Tight Junction Permeability across MDCK Cell Monolayers. U73122, a PLC-β inhibitor (Bleasdale et al., 1989) that is structurally unrelated to alkylphosphocholines (Fig. 5A), decreased ATP-stimulated PLC-β activity and TEER in a concentration-dependent manner: $IC_{50(PLC)}$ and EC_{50} were estimated to be 5 ± 3 μM and 6 ± 2 μM, respectively (Fig. 5B; Table 1). Thus U73122 appears to be one of the most potent inhibitors of PLC-β and enhancers of tight junction permeability identified thus far (Table 1). Interestingly, the relationship between the potencies of U73122 to increase tight junction permeability and inhibit PLC-β was the same as that observed for alkylphosphocholines; this was evident by the fact that the $IC_{50(PLC)}$, EC_{10x}, and EC_{50} values for U73122 fitted well in the plot of $IC_{50(PLC)}$ versus EC_{10x} or

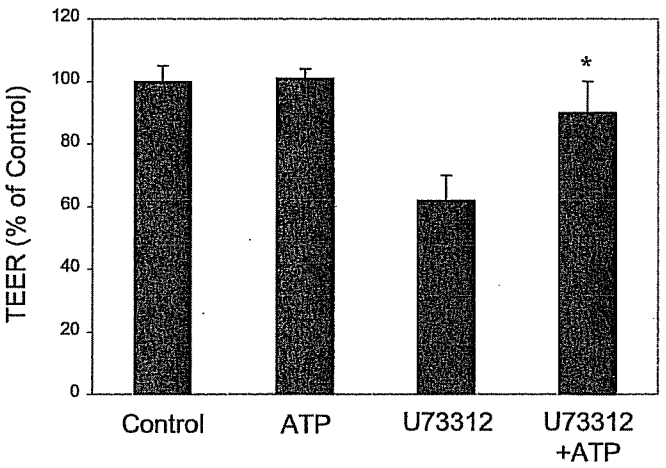

Fig. 6. Effect of ATP on U73122-induced decrease in TEER. U73122 (5 μM) with or without ATP (100 μM) was added to the apical compartment. TEER was measured after a 30-min incubation at 37°C. The asterisk denotes a significant difference ($p < 0.05$) between TEER of MDCK cell monolayers treated with U73122 and with U73122 plus ATP.

EC_{50} for alkylphosphocholines—r values improved from 0.969 to 0.982 (EC_{10x}) and from 0.901 to 0.947 (EC_{50}) by inclusion of U73122 in the correlation (Fig. 4). U73343, the structural analog of U73122 (Fig. 5A), shown to be inactive as an inhibitor of PLC (Bleasdale et al., 1989), showed no effect on tight junction permeability (Fig. 5B). These results showed that even when PLC-β inhibitors are structurally different, the relationship between inhibition of the enzyme and enhancement of the tight junction permeability remains intact. This represents more definitive evidence for a relationship between inhibition of PLC-β and enhancement of tight junction permeability. Further confirmation of this hypothesis was obtained from the observation that reversing the inhibition of PLC-β could significantly attenuate the enhancement of tight junction permeability by U73122. The reversal of PLC-β inhibition was achieved by treatment of the cells with excess ATP (Fig. 6), which can activate PLC-β via Ġ protein-coupled receptors (e.g., $P2Y_2$ receptors) (Katan, 1998).

Effect of HPC and U73122 on Actin Filament Organization. In searching for the cellular event that accompanied enhanced tight junction permeability caused by inhibitors of PLC-β (e.g., HPC and U73122), we observed that the organization of actin was affected by these compounds. The confocal microscopic images of the untreated (control) cells revealed the presence of actin filaments (visualized with phalloidin-conjugated Texas Red) at cortical regions (i.e., mid-cell level) around the cell border (Fig. 7); this cortical actin ring has been previously observed (Hirokawa and Tilney, 1982). HPC and U73122 induced the disorganization of these filaments, as evidenced by the presence of actin aggregates in the cytoplasmic space (Fig. 7). The potency of the effect of HPC and U73122 on actin organization was consistent with their potency as enhancers of tight junction permeability. For example, HPC had no affect on tight junction permeability and actin filament organization at 10 μM but decreased TEER by 60% and induced a marked disorganization of actin filaments in some cells of the monolayer at 30 μM, as evidenced by the presence of actin in the cytoplasm near the cortical region, outlining the nucleus (Fig. 7A). A

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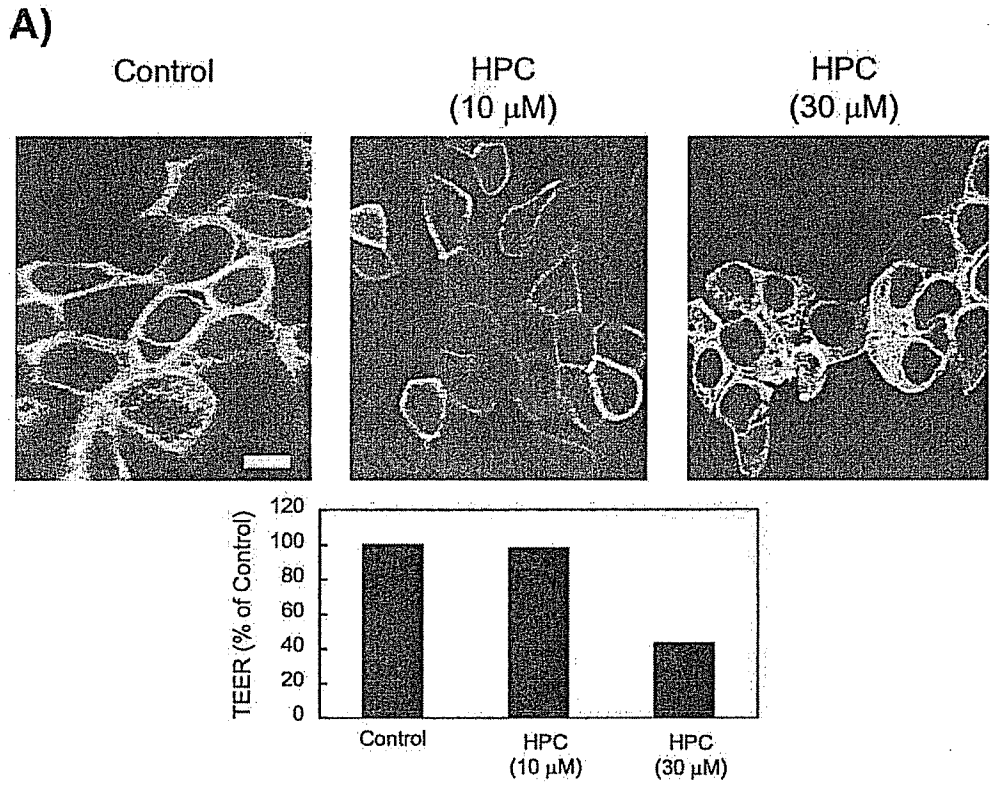

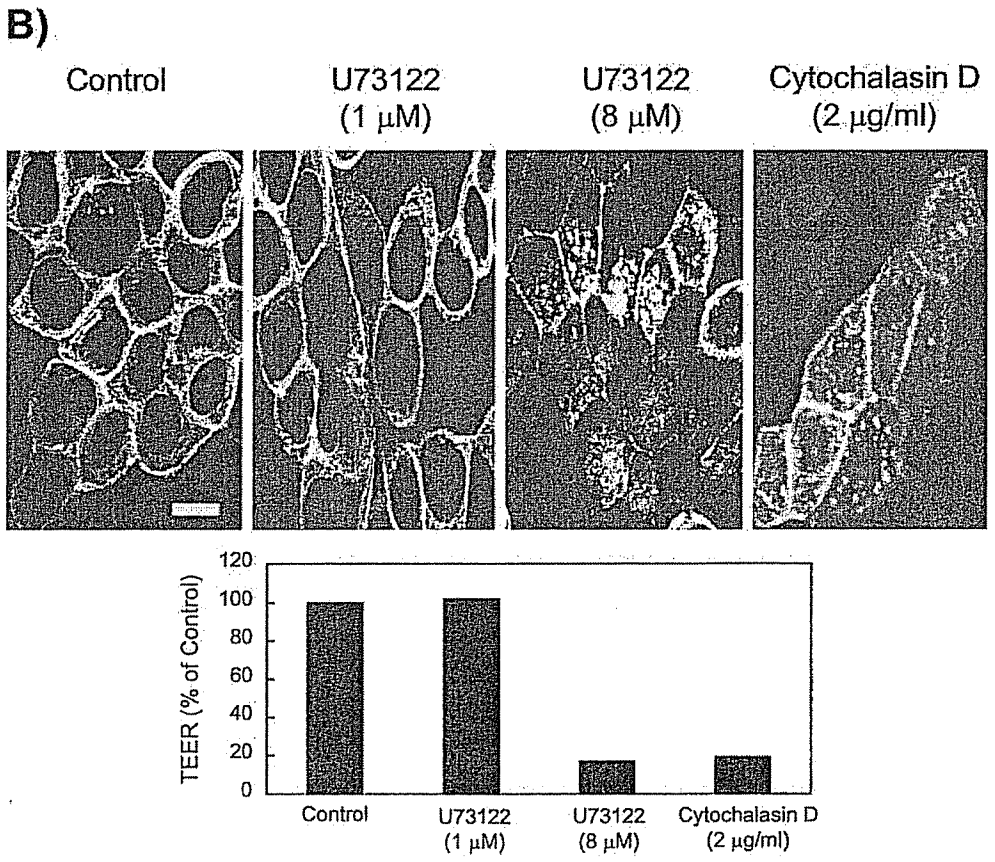

Fig. 7. Effect of HPC (A) and U73122 (B) on actin filament organization of MDCK cell monolayers. The cells were treated apically with HPC or U73122 for 30 min at 37°C. TEER was measured before and after treatment. Cell monolayers were then incubated with phalloidin conjugated with Texas Red in 3.7% formaldehyde for 30 min at 4°C and subsequently washed with transport buffer. Actin filament organization was viewed with a Zeiss confocal microscope. The bar denotes 20 μm.

similar concentration-dependent increase in tight junction permeability and disorganization of actin filaments was caused by U73122 (Fig. 7B). At 8 μM, U73122 induced punctate actin aggregates in the cytoplasm and markedly decreased TEER (Fig. 7B), which was similar to the effect observed by cytochalasin D, a compound that directly disrupts actin cytoskeleton organization (Stevenson and Begg, 1994). It is important to note that organization of the apical actin, unlike that of cortical actin, was unaffected by treatment of cells with relevant concentrations of U73122 and HPC (data not shown). These results suggest that inhibition of PLC-β induces an increase in tight junction permeability specifically via disorganization of the cortical actin filaments.

Attenuation of U73122-Mediated Disorganization of Actin Filaments by ATP. Since ATP can attenuate U73122-induced increase in tight junction permeability by reversing the inhibition of PLC-β (Fig. 6), its effect on U73122-induced disorganization of actin filaments was assessed. At 5 μM, U73122 decreased TEER by 40% and induced a marked disorganization of actin filaments (Fig. 8). Interestingly, the effect of U73122 on tight junction permeability and organization of actin was markedly diminished after cotreatment with ATP (100 μM) (Fig. 8). ATP alone had a negligible effect on tight junction permeability and organization of actin filaments (Fig. 8).

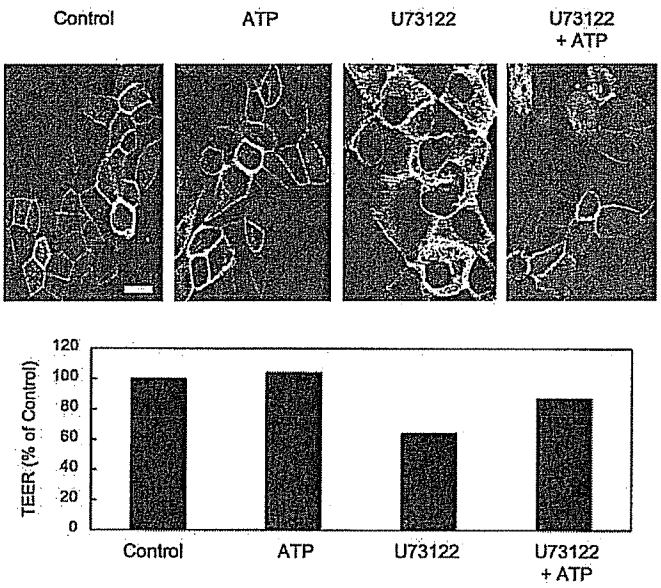

Fig. 8. Attenuation of U73122-mediated disorganization of actin filaments in MDCK cell monolayers by ATP. The cells were treated with U73122 (5 μM) with or without ATP (100 μM) for 30 min at 37°C. TEER was measured before and after treatment. The actin filaments were visualized as described in Fig. 7. The bar denotes 25 μm.

Discussion

In MDCK cell monolayers, the PLC-dependent pathway has been implicated in the assembly of the tight junction (Cereijido et al., 1993a). For example, activation of PLC by thyrotropin-1-releasing hormone stimulated tight junction assembly, and its inhibition by neomycin blocked this assembly in MDCK cells (Balda et al., 1991). Diacylglycerol is a product of the reaction catalyzed by PLC. An analog of diacylglycerol, i.e., 1,2-dioctanoylglycerol, stimulated tight junction assembly via activation of the downstream enzyme PKC (Balda et al., 1991). Interestingly, knockout of the gene coding for the PLC isozyme, PLC-β3, in mice produced an embryonic lethal mutant in which the blastocoele failed to develop (Wang et al., 1998). Similarly, in Drosophila, PLC-γ is expressed predominantly in the blastoderm cell (Emori et al., 1994), and this PLC isozyme is involved in the Drosophila development disorder known as small wing (Thackeray et al., 1998). In light of the evidence that the tight junction formation is essential for development (i.e., blastocoele cavitation) (Fleming et al., 2000), these findings may point to the potential importance of PLC in the assembly of tight junctions during development. In contrast, the role of the PLC-dependent pathway in the regulation of the tight junction structure and function in mature epithelial tissues is not clearly understood.

In the present study, we have utilized a homologous series of alkylphosphocholines to produce systematic changes in the activity of the PLC-β isozyme and in the tight junction permeability across MDCK cell monolayers. The basal PLC activity in MDCK cells was quite low, presumably comprised of multiple PLC isozymes. PLC-β was selectively activated (versus PLC-γ) by treatment of the cells with ATP. The selective PLC-γ inhibitor, 3-nitrocoumarin, did not affect ATP-stimulated PLC activity, thus confirming the absence of significant PLC-γ activity in these cells. As reported previously (Nicholas et al., 1996), ATP activates PLC-β via the G-protein-coupled receptor $P2Y_2$. The separation between the basal levels of [^{3}H]inositol phosphates and those in ATP-stimulated cells was not sufficiently high (>3 fold) to allow measurement of PLC-β activity and its inhibition. Thus the effect of these compounds was measured on ATP-stimulated PLC activity in MDCK cells that were transfected with a plasmid encoding the $P2Y_2$ receptor. The $P2Y_2$-transfected MDCK cells are identical to the native cells in all respects except for their greater $P2Y_2$ receptor activity. A relationship ($r > 0.90$, $p < 0.05$) was observed between the potency of alkylphosphocholines as inhibitors of ATP-stimulated PLC-β and their potency as enhancers of tight junction permeability in MDCK cell monolayers. Such a relationship between the inhibition of PLC-β activity and tight junction permeability, observed for a series of homologues, provided more unequivocal evidence for the role of PLC-β in the modulation of tight junction permeability than would be possible with the use of a single inhibitor or activator. The use of a homologous series of compounds, instead of a single agent as a mechanistic tool, makes it less likely, although not impossible, to mistakenly infer that a relationship exists between two apparently unrelated events. Furthermore, U73122, a PLC-β inhibitor that is structurally unrelated to alkylphosphocholines, inhibited PLC-β and enhanced tight junction permeability in MDCK cell monolayers with relative potencies that were entirely consistent with those of alkylphosphocholines. In contrast, U73343, a structural analog of U73122, which is inactive as a PLC inhibitor, was also found to be inactive as an enhancer of tight junction permeability (Fig. 5B). This observation provided further confirmation for the relationship between PLC-β activity and tight junction permeability, because the probability is very small that two structurally diverse compounds (or classes of compounds) would affect PLC-β activity and tight junction permeability at relevant concentrations through totally unrelated mechanisms. Finally, activating

this enzyme with the purinergic receptor agonist ATP significantly attenuated inhibition of PLC-β by U73122, with a concurrent reversal of the enhancement of the tight junction permeability. Together, these results construct strong evidence that PLC-β activity can functionally affect the tight junction permeability of an epithelial tissue. It is important to note that HPC (alkylphosphocholines) and U73122 are selective inhibitors of PLC-β and do not inhibit EGF-stimulated PLC-γ (Ward et al., 2002), because inhibition of PLC-γ by 3-nitrocoumarin also leads to enhancement of tight junction permeability (Ward et al., 2002).

Our observation that these PLC-β inhibitors caused disorganization of actin filaments at concentrations that enhanced the tight junction permeability suggested that the enhancement in the tight junction permeability is caused by modulation of the tight junction architecture. That ATP attenuated the effect of these agents on actin organization and on PLC-β activity, is indicative of a specific mechanism-based association between the two events rather than nonspecific toxic events following inhibition of the enzyme. This is consistent with the previous observation that tight junction permeability can be regulated by actin-containing cytoskeleton (Anderson and Van Itallie, 1995). It is interesting to note that inhibition of PLC-γ by 3-nitrocoumarin leads to qualitatively different effect on the cortical actin filament in that a punctate staining for actin is observed at intercellular junctions, unlike the presence of actin aggregates in the cytoplasmic space observed upon inhibition of PLC-β (Ward et al., 2002).

The actin cytoskeleton associates with the tight junction and plasma membrane, specifically through a network of actin filaments underneath the tight junction and through a cortical ring of actin filaments at the level of the adherens junction (i.e., perijunctional actin myosin ring) (Hirokawa and Tilney, 1982). In guinea pig ileum, disruption of the actin cytoskeleton by drugs, such as cytochalasin D, increased sodium and mannitol flux (Madara, 1986), suggesting the importance of the integrity of the cytoskeleton on the function of junctional complexes. PLC-β may alter the organization of cortical actin filaments through regulation of PKC. The involvement of PKC has been implicated in zonula occludens toxin-induced disorganization of actin filaments and increased tight junction permeability (Fasano et al., 1995). Furthermore, the actin binding protein, vinculin, is a target of PKC phosphorylation during junctional assembly induced by calcium (Perez-Moreno et al., 1998). Rho may also be involved in this pathway, since inhibition of rhoA with C3 transferase induced disassembly of the cytoskeletal actin, including the perijunctional actin myosin ring, and increased tight junction permeability (Nusrat et al., 1995). Furthermore, U73122 was found to inhibit Rho activity (Nozu et al., 1999).

The present study provides early insights into how epithelial tight junctions could be regulated via receptor-initiated cellular signaling events. PLC-β, a regulatory enzyme, emerges as an important player in the regulation of structure and function of epithelial tight junctions. Clearly, discrete events that mediate changes in the tight junction structure and function following changes in the PLC-β activity remain to be elucidated. Such studies will lead to a better understanding of the cellular regulation underlying the barrier function of the epithelial and endothelial tissues.

Acknowledgments

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